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AROMATIC AMINO ACID CATABOLISM BY *LACTOBACILLUS SPP.*:
BIOCHEMISTRY AND CONTRIBUTION TO CHEESE FLAVOR DEVELOPMENT

by

Sanjay Gummalla

A dissertation submitted in partial fulfillment
of the requirements for the degree

of

DOCTOR OF PHILOSOPHY

in

Nutrition and Food Sciences

Approved:

UTAH STATE UNIVERSITY
Logan, Utah

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ABSTRACT

Aromatic Amino Acid Catabolism by *Lactobacillus* spp.: Biochemistry
and Contribution to Cheese Flavor Development

by

Sanjay Gummalla, Doctor of Philosophy

Utah State University, 2002

Major Professor: Dr. Jeffery R. Broadbent
Department: Nutrition and Food Sciences

Amino acids derived from the degradation of casein in cheese serve as precursors for the generation of desirable and undesirable flavor compounds. Microbial degradation of aromatic amino acids is associated with the formation of aroma compounds that impart putrid-fecal, barny-utensil, and floral off-flavors in cheese, but pathways for their production had not been established. This study investigated Tyr and Phe catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts under simulated Cheddar cheese-ripening conditions (pH 5.2, 4% NaCl, 15°C, no sugar). Enzyme assays of cell-free extracts and micellar electrokinetic capillary chromatography of supernatants indicated that *L. casei* and *L. helveticus* strains catabolize Tyr and Phe by successive transamination and dehydrogenation reactions. Major products of Tyr and Phe catabolism included off-flavor compounds formed by chemical degradation of the α -keto acids, produced by transamination, and aromatic α -hydroxy acids derived from α -keto acids by α -hydroxy acid dehydrogenases. Action of *Lactococcus lactis*

aminotransferase enzymes on Trp, Tyr, and Phe also leads to the formation of α -keto acids, but unlike lactobacilli, the former bacteria do not express dehydrogenase activity under cheese-like conditions (pH 5.2, 4% NaCl, 15°C, no sugar). Since aromatic α -keto acids may degrade spontaneously into undesirable flavor compounds, α -hydroxy acid dehydrogenases may be useful in controlling off-flavor development via diversion of chemically labile α -keto acids to more stable α -hydroxy acids. To test this hypothesis, we investigated the effect of D-hydroxyisocaproate dehydrogenase overexpression by a *L. casei* adjunct on chemical and sensory properties of reduced-fat Cheddar cheese made with and without addition of 20 mM α -ketoglutarate. The D-hydroxyisocaproic acid dehydrogenase gene (*D-HicDH*) was cloned into a high copy number vector pTRKH2 and transformed into *L. casei* ATCC334. Reduced-fat Cheddar cheeses were made with *Lactococcus lactis* starter only, starter + *L. casei* ATCC334 with pTRKH2, and starter + *L. casei* ATCC334 with pTRKH2: *D-HicDH*, and then volatile analysis was performed by gas chromatography and mass spectrometry. Statistical analysis of volatile data after 3 mo of ripening at 7°C showed profiles of ketones, aldehydes, alcohols, esters, sulfur compounds, and benzaldehyde were significantly altered by culture treatments and α -ketoglutarate addition, and these treatments also affected sensory flavor attributes of experimental cheeses. Results also indicated overexpression of D-hydroxyisocaproic acid dehydrogenase can divert labile α -keto acids into more stable compounds, but the overall effect seemed to diminish both beneficial and detrimental flavor notes.

To Amma and Daddy

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LIST OF ABBREVIATIONS

AAA	=Aromatic amino acids
ATase	=Aminotransferase
BA	=Benzoic acid
BCAA	=Branched chain amino acids
CBL	=Cystathionine- β -lyase
CDM	=Chemically defined medium
CFE	=Cell free extract
CGL	=Cystathionine- γ -lyase
DCOOHase	=Decarboxylase
D-HicDH	=D-Hydroxyisocaproate dehydrogenase
Em	=Erythromycin
GC-MS	=Gas chromatography-Mass spectrometry
HA-DH	=Hydroxyacid dehydrogenase
HPAA	= ρ -Hydroxyphenylacetic acid
HPLA	= ρ -Hydroxyphenyllactic acid
HPLDHase	= ρ -Hydroxyphenyllactate dehydrogenase
HPPA	= ρ -Hydroxyphenylpyruvic acid
IAA	=Indole acetic acid
IPA	=Indole pyruvic acid
ILA	=Indole lactic acid
KG	= α -ketoglutarate

KMBA=4-methylthio-2-oxobutyric acid

LAB=Lactic acid bacteria

MECC=Micellar electrokinetic capillary chromatography

NCR=Near-cheese ripening

NSLAB=Non-starter lactic acid bacteria

PPA=Phenylpyruvic acid

PLA=Phenyllactic acid

PLDHase=Phenyllactate dehydrogenase

PLP=Pyridoxal-5-phosphate

CHAPTER I

INTRODUCTION

Cheddar is a highly prized variety of cheese in North America, Europe, and Australia. In the United States, Cheddar varieties account for a significant proportion of total cheese consumed (Gorski, 1997). In recent years, the popularity of Cheddar cheese has expanded to encompass lower fat varieties and the lack of typical cheese flavor in these varieties has stimulated renewed interest in cheese flavor research. In Cheddar cheese, flavor development occurs during the ripening phase, where cheese is held between 4 to 12°C for a period that ranges from 3 mo to a year or more. During this time, enzymes from milk, added coagulant, and bacteria break down milk carbohydrates, fats, and proteins. Although glycolysis and lipolysis are integral to the ripening process, proteolysis and its secondary reactions are widely thought to have the most important effects on cheese texture and flavor development. The works of Christensen et al. (1995) and McGarry et al. (1995) showed the link between proteolysis and cheese flavor was not directly due to the release of free amino acids. This finding was also supported by Wallace and Fox (1997), who suggested aroma formation was not limited by release of free amino acids, but instead by ensuing secondary metabolism of amino acids and subsequent production of flavor compounds. Yvon et al. (1998) validated this hypothesis by demonstrating that α -ketoglutarate, a key reactant in amino acid metabolism by lactic acid bacteria (**LAB**), was the rate-limiting factor for cheese aroma development.

As a result, amino acid catabolism by LAB and its role in cheese flavor development have become major areas of research. Degradation of aromatic amino (**AAA**) has received special attention because these reactions have been implicated in the

production of off-flavors. Specifically, AAA catabolism has been linked to the generation of aroma compounds that impart unclear, medicinal, fecal, and rosy off-flavors to cheese (Guthrie, 1993). Such off-flavors are particularly problematic in reduced-fat varieties (Milo and Reineccius, 1997).

Studies of AAA catabolism by LAB have primarily focused on the Cheddar cheese starter bacterium *Lactococcus lactis*, and on *Lactobacillus* spp. that are used as cheese flavor adjuncts or which enter cheese as adventitious contaminants from milk or processing equipment (Christensen et al., 1999; Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999; Rijnen et al., 1999a; Yvon et al., 1997; Yvon et al., 1998; Yvon and Rijnen, 2001). Gao et al. (1997) showed that *Lactococcus lactis* produced an AAA aminotransferase (**ATase**) that converted AAA into their corresponding α -keto acids under cheese ripening conditions (pH 5.2, 4 % NaCl, 13°C). Those authors also showed that non-viable starter bacteria in the cheese matrix may contribute to amino acid catabolism, and suggested that inter-conversion of AAA metabolites by starters and lactobacilli could lead to the production of off-flavor compounds. Yvon et al. (1997) purified and characterized the lactococcal AAA ATase and subsequently reported that St. Paulin cheese made with a starter that lacked this enzyme had a lower level of AAA degradation and floral-like off-flavors (Rijnen et al., 1999b).

Current molecular understanding of AAA degradation in dairy lactobacilli is not as advanced as it is for *Lactococcus lactis*, but biochemical data indicate that AAA catabolism in lactobacilli is almost always initiated by an aminotransferase (Groot et al., 1998; Gummalla and Broadbent, 1999). Groot et al. (1998) showed that *Lactobacillus*

plantarum, a species that is commonly found among cheese nonstarter lactic acid bacteria (**NSLAB**) (Peterson and Marshall, 1990), converts Phe to phenylpyruvic acid (**PPA**) by transamination, and that PPA may degrade spontaneously into benzaldehyde. Our laboratory investigated Trp catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts under simulated cheese-ripening conditions (pH 5.2, 4% NaCl, 13°C, no sugar) and showed these species catabolized Trp to indole lactic acid (**ILA**) via successive transamination and dehydrogenation reactions (Gummalla and Broadbent, 1999). Like PPA, the α -keto acid intermediate formed by Trp transamination, indole pyruvic acid (**IPA**), was chemically labile and could degrade spontaneously into a variety of other aromatic compounds (Gao et al., 1997; Gummalla and Broadbent, 1999). Since α -hydroxy acids such as ILA have no significant effect on cheese flavor, the ability of lactobacilli to enzymatically reduce α -keto acids may provide a mechanism to control off-flavors that arise by chemical decomposition of α -keto acids produced by other cheese bacteria.

Given that catabolism of AAA and their metabolites by starter, adjunct, and NSLAB is an important source of off-flavors in cheese, detailed knowledge of AAA catabolic pathways in these organisms should reveal useful strategies to control aromatic off-flavor development in Cheddar cheese. The work of Rijnen et al. (1999b) with AAA ATase-negative starter bacteria provides encouragement for industry efforts to control cheese off-flavor development, but full realization of this goal will clearly require a more comprehensive understanding of AAA catabolism by lactobacilli and perhaps other bacteria that occur in ripened cheese.

During the research outlined in this dissertation, I investigated pathways for catabolism of Tyr and Phe in *L. casei* and *L. helveticus* cheese flavor adjuncts under cheese-ripening conditions. That work was followed by experiments to investigate the effect of D-hydroxyisocaproate dehydrogenase (**D-HicDH**) overexpression in a *L. casei* adjunct on chemical and sensory properties of reduced-fat Cheddar cheese.

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CHAPTER II

LITERATURE REVIEW

Lactic acid bacteria (**LAB**) are a diverse group of Gram-positive, non-sporing, catalase-negative, microaerophilic, and nutritionally fastidious microorganisms that produce lactic acid as a primary product of glucose fermentation (Salminen and von Wright, 1993). These bacteria occupy a variety of environments including raw agricultural commodities, fermented foods, the oral cavity, and the intestinal and reproductive tracts of humans and animals. The LAB also have significant industrial value because they are essential for the manufacture of a variety of fermented dairy, meat, vegetable, and cereal foods. In addition, some species of LAB are thought to have human probiotic (health-promoting) and nutritional benefits.

Cheddar Cheese Flavor

Cheddar is a semi-hard ripened cheese variety that is usually manufactured using a defined starter comprised of one or more strains of the LAB species *Lactococcus lactis*. During cheese ripening, starter lactococci slowly die off and adventitious nonstarter lactic acid bacteria (**NSLAB**), which enter cheese through milk or processing equipment, grow to levels of 10^7 to 10^9 per gram. In most Cheddar cheese, the NSLAB population is dominated by lactobacilli (Peterson and Marshall, 1990). The absence of Cheddar flavor development in aseptic, directly acidified cheese showed starter, NSLAB, and their enzymes were required for proper cheese flavor development (Reiter et al., 1967). Additionally, some species of *Lactobacillus* are used as adjunct cultures to intensify flavor development in Cheddar cheese (El Soda, 1993). Although addition of lactobacilli

has been shown to enhance cheese flavor intensity and to accelerate ripening, these bacteria sometimes promote flavor defects (Khalid and Marth, 1990; Laleye et al., 1990; Lee et al., 1990).

Typical Cheddar flavor is characterized by a pleasant, sweet, and aromatic walnut sensation devoid of any single note (Kosikowski, 1966). To attain characteristic flavor, freshly pressed Cheddar curd is aged between 4-12°C for at least 3 mo and sometimes for as long as a year or more. In cheese aged for longer periods, an acrid quality renders cheese sharp (Kosikowski, 1966). At the onset of ripening, starter metabolism of cheese peptides, carbohydrates, and fat initiates many reactions of flavor development (Cogan, 1995). During later months of maturation, enzymes released by starter autolysis continue to catalyze these reactions, supplying precursors that may be converted to flavor compounds by growing populations of NSLAB (Adda et al., 1982).

Early theories suggested that characteristic Cheddar flavor was the result of a single compound or class of compounds, but efforts to identify these molecules were not successful (Aston and Dulley, 1982). Some researchers, however, have attributed Cheddar flavor to a strong sulfur note that is associated with the production of methional (Adda et al., 1982; Aston and Dulley, 1982; Law, 1981). The contemporary and more widely accepted hypothesis for Cheddar flavor development is the component balance theory (Mülder, 1952). It states that Cheddar flavor requires the right proportions of numerous compounds that include amines, amino acids, carbonyls, fatty acids, peptides, and sulfur derivatives. These compounds emerge from the primary breakdown of milk constituents (carbohydrates, lipids, and proteins) and from subsequent secondary reactions that occur during cheese maturation.

Flavor Defects of Reduced-Fat Cheese

Flavor of reduced-fat cheese is noticeably different from that of a full-fat variety (Mistry, 2001). Reduced-fat Cheddar suffers from poor flavor intensity and an increased propensity for off-flavors including unclean, medicinal, meat brothy, and fecal notes (Guthrie, 1993). Although demand for low fat cheeses has risen in recent years, these negative attributes have resulted in declining consumer acceptance of these types of cheeses (Mistry, 2001). The chemical basis for low Cheddar flavor intensity in reduced-fat varieties remains unclear, but some authors have attributed it to decreased amounts of specific fatty acids and lipolytic products like methyl ketones (Adda et al., 1982; Banks et al., 1989; Foda et al., 1974; Ohren and Tuckey, 1969; Reiter et al., 1967). Flavor problems in reduced-fat cheeses have also been attributed to differences that may exist in the rate of release of flavors from cheese, thereby altering flavor perception (Laloy et al., 1996; Milo and Reineccius, 1997; Mistry, 2001). Alternatively, because reduced-fat cheeses typically replace fat with water, and flavor thresholds of many compounds are usually lower in water than in fat (Forss and Patton, 1966), flavor defects such as meaty brothy or unclean flavors may result from accumulation of fat-soluble flavor compounds in the water phase. Since many off-flavor compounds arise from aromatic amino acid (AAA) catabolism by bacteria in the cheese matrix, an improved understanding of AAA catabolism by cheese bacteria may reveal strategies to control off-flavor defects by reducing levels of these compounds in low fat cheese.

Proteolysis and Flavor Development

While enzymatic reactions of glycolysis and lipolysis will have an influence on Cheddar cheese flavor, proteolysis and its secondary reactions are widely believed to play a much more significant role in flavor development (Fox, 1989; Fox et al., 1994).

Research has demonstrated that primary hydrolysis of caseins is catalyzed by the added coagulant and to a lesser extent, the native milk proteinase plasmin. Subsequent conversion of large and medium-sized peptides produced by those enzymes into small peptides and free amino acids in cheese is achieved by the action of microbial proteinases and peptidases (Fox et al., 1994). With the exception of bitter peptides (Broadbent et al., 2002; Broadbent et al., 1998; Edwards and Kosikowski, 1983; Stadhouders et al., 1983), low molecular mass oligopeptides have no direct flavor contribution, but free amino acids derived from these peptides serve as precursors for the generation of key flavor compounds in cheese (Christensen et al., 1995; Christensen et al., 1999; Hemme et al., 1982; Gao et al., 1997; Urbach, 1995). While the specific nature of these reactions and their influence on flavor development remains speculative, many investigators believe catabolism of amino acids by LAB may be required for cell survival in the inclement environment of ripening cheese (low pH, high salt, and low temperature). This hypothesis is based on the knowledge that starter metabolism consumes residual lactose in cheese during the first few weeks of ripening, so bacteria must utilize other sources of carbon and nitrogen for growth or survival.

Amino Acid Catabolism and Cheese Flavor

Research on the contribution of amino acid catabolism to cheese flavor and aroma suggests degradation of Met, branched chain amino acids (BCAA), and AAA are particularly important for production of alcohols, aldehydes, ketones, esters, and sulfur compounds in cheese. For example, production of methional and methanethiol from Met, and 3-methylbutanal and isovaleric acid from Leu, impart potent sulfurous and malty odorants, respectively, in many types of cheese (Yvon and Rijnen, 2001). In addition, isobutyric acid from Val catabolism contributes sweet and pleasant flavors to cheese (Weimer et al., 1999; Yvon and Rijnen, 2001). Unlike Met and BCAA catabolism, microbial degradation of Trp, Tyr, and Phe may lead to the formation of persisting unclean flavors in cheese (Christensen et al., 1999; Dumont and Adda, 1978; Lindsay, 1992; Parliament et al., 1982; Schormüller, 1968; Yokoyama and Carlson, 1981).

Specifically, the Phe catabolites phenyl acetaldehyde and phenethanol caused floral or rose-like off-flavors, and the Tyr catabolite *p*-cresol imparted barny or utensil-like off-flavors (Dunn and Lindsay, 1985). Guthrie (1993) also showed AAA metabolites such as *p*-cresol (from Tyr) and indole (from Trp) imparted utensil and putrid, fecal flavors to Cheddar cheese, and suggested these compounds were produced by cheese starter bacteria and NSLAB. The presence of indole in the aroma concentrate of *Lactobacillus helveticus* cultures and cheeses provides additional evidence that AAA catabolism by cheese bacteria may be an important source of aromatic off-flavor (Kowaleska et al., 1985). Although pathways for the production of each of these molecules from AAA have been described in some bacteria (Macfarlane and Macfarlane, 1995), mechanisms for their production in cheese have not been established.

Nonetheless, the ability to catabolize amino acids to aroma compounds has been confirmed in many LAB including lactococci, lactobacilli, and carnobacteria (Williams et al., 2001; Yvon and Rijnen, 2001). As noted above, most research has focused on the catabolism of Met, BCAA, and AAA because of their importance to flavor of dairy products. Evidence to date indicates that the primary mechanism for amino acid catabolism by these species is transamination by one or more aminotransferase (**ATase**) (Ayad et al., 1999; Dias and Weimer, 1998b; Gao et al., 1998; Kieronczyk et al., 2001). Transamination of Met, for example, yields 4-methylthio-2-oxobutyric acid (KMBA), which is chemically labile and can degrade spontaneously to methanethiol (Gao et al., 1998). Methanethiol is a precursor of important flavor compounds in cheese, such as dimethyldisulfide and dimethyltrisulfide (Parliament et al., 1982). Side chain elimination reactions such as Met γ -elimination may also be important to cheese flavor because they can facilitate production of methanethiol (Weimer et al., 1999; Yvon and Rijnen, 2001).

In LAB, Met γ -elimination reactions are catalyzed by cystathionine β -lyase (**CBL**) cystathionine γ -lyase (**CGL**) (Alting et al., 1995; Bruinenberg et al., 1997). The native role of these enzymes involves β - and γ -eliminations, respectively, of cystathionine, but Met γ -elimination is also catalyzed at lower efficiency (Dias and Weimer, 1998a; Smacchi and Gobbetti, 1998). Characterization of the CBL and CGL enzymes of *Lactococcus lactis* has indicated that their primary role is more likely concerned with Met anabolism rather than Met catabolism (Alting et al., 1995; Bruinenberg et al., 1997). However, overexpression in *Lactococcus lactis* of *metC*,

which encodes CBL, can increase production of volatile sulfur compounds (Fernandez et al., 2000).

Like Met catabolism, BCAA metabolism in LAB is typically initiated by ATase-mediated transamination (Atilas et al., 2000; Hansen et al., 2001; Yvon and Rijnen, 2001). The BCAA aminotransferases from two strains of *Lactococcus lactis* have been characterized and shown to be active under cheese ripening conditions (Atilas et al., 2000; Yvon et al., 2000). Both enzymes showed highest activity toward BCAA substrates, but were also active on Met and AAA. In contrast, the BCAA ATase of *Lactobacillus paracasei* was specific for BCAA and exhibited no ATase activity with AAA or Met (Hansen et al., 2001).

Aromatic Amino Acid Catabolism among LAB

Research has demonstrated that starter lactococci and adjunct lactobacilli incubated under cheese-like conditions are each able to convert Trp to indole pyruvic acid (IPA) via transamination by ATase (Gao et al., 1997; Gummalla and Broadbent, 1999). Both AAA ATase and Asp ATase are able to catalyze the above reaction in *Lactococcus lactis* (Yvon and Rijnen, 2001). As has been noted with other α -keto acid intermediates, IPA may undergo spontaneous degradation to indole 3-aldehyde and indole acetic acid (IAA) (Gao et al., 1997; Gummalla and Broadbent, 1999).

These secondary non-enzymic reactions may be important to off-flavor development because some lactobacilli can convert IAA to skatole, a compound that imparts very unclean flavor to cheese (Guthrie, 1993; Yokoyama and Carlson, 1981). Unlike starter bacteria, however, lactobacilli were able to enzymatically convert IPA to

indole lactic acid (**ILA**), which has no impact on cheese flavor. This observation suggested lactobacilli might be used to restrict the production of IAA generated from IPA produced by starter bacteria (Gummalla and Broadbent, 1999). It was therefore our hypothesis that new strategies to improve flavor development in reduced-fat cheese would emerge from a better understanding of AAA catabolism by cheese bacteria.

In most bacteria, enzymes for Trp, Tyr, and Phe catabolism can be divided into three groups (Macfarlane and Macfarlane, 1995): lyases, decarboxylases, and transaminases. Action of AAA lyases results in the removal of the side chain and liberation of the aromatic nucleus. For example, Tyr phenol lyase (EC 4.1.99.2) catalyzes the single step hydrolysis of Tyr to phenol. Phe lyase activity is not reported in bacteria but the theoretical product of this reaction is benzene. The above elimination reactions of AAA are not known to occur in cheese bacteria, but both phenol and benzene have been detected in cheese (Guthrie, 1993). Trp indole lyase (EC 4.1.99.1) or tryptophanase is a pyridoxal 5-phosphate (**PLP**) dependent enzyme that cleaves Trp to produce indole, ammonia, and pyruvate. The prevalence of indole in some cheeses and its potential to cause fecal off-flavors has motivated investigations to reveal the mechanism(s) for its production in cheese. Although evidence for the ability of some LAB to produce indole via this pathway has been uncovered, conclusive proof for tryptophanase activity in LAB has not been provided (Gummalla and Broadbent, 1996).

The Tyr (EC 4.1.1.25), Phe (EC 4.1.1.53), and Trp (EC 4.1.1.28) decarboxylases catalyze the conversion of Tyr to tyramine, Phe to phenethylamine, and Trp to tryptamine, respectively (Nakazawa et al., 1977). Although aromatic amines do not have a significant impact on cheese flavor, their biogenic effects raise important health

concerns among cheese consumers (Kowaleska et al., 1985). In addition, aromatic amines can be converted to aromatic acetic acids via an amine oxidase (EC 1.4.3.4), or reduced to aromatic ethanols that can contribute floral off-flavors to cheese (Yuasa et al., 1976). Decarboxylase activity among LAB is variable; Gao et al. (1997) reported no AAA decarboxylase in lactococci, but Trp decarboxylase was detected in cell-free extracts of *L. casei* and *L. helveticus* cheese flavor adjuncts (Gummalla and Broadbent, 1999). In the latter study, however, the product of Trp decarboxylation, tryptamine, was not identified in culture supernatants. Tyramine production has been reported for *Lactobacillus brevis*, *Lactobacillus buchneri*, and *Lactobacillus fermenti* (Hupfer et al., 1950) and other studies have found limited decarboxylase activity in various species of lactobacilli (Guthrie, 1993; Joosten and Northolt, 1987; Moreno-Arribas et al., 2000).

Transamination reactions facilitate the removal of nitrogen and can be catalyzed by three distinct types of enzymes: AAA dehydrogenase, AAA oxidase, and AAA ATase. Many bacteria produce Phe dehydrogenase (EC 1.4.1.20), but this enzyme is not known to occur in LAB (Hummel et al., 1984; Hummel et al., 1986). The presence of AAA oxidases in LAB is also unlikely, because these enzymes require cytochromes, and LAB lack cytochromes because they cannot synthesize Heme (Salminen and von Wright, 1993). For these reasons, ATase (EC 2.6.1.1) provide the primary mechanism for amino acid catabolism by LAB (Gao et al., 1997; Gummalla and Broadbent, 1999; Rijnen et al., 1999a; Yvon et al., 1997). ATase are PLP dependent and require an α -keto acid acceptor, such as α -ketoglutarate (Yvon et al., 1998). They generally display preferential specificity toward a class of related amino acids (AAA or BCAA), but many will catalyze

transamination of a broader range of substrates. ATase-mediated transamination reactions are reversible and can play a role in both catabolic and anabolic reactions.

Gao et al. (1997) reported that *Lactococcus lactis* initiated breakdown of AAA by an ATase-catalyzed reaction. The lactococcal AAA ATase has been purified from *Lactococcus lactis* ssp. *lactis* S3 (Gao and Steele, 1998) and from *Lactococcus lactis* ssp. *cremoris* NCDO763 (Yvon et al., 1997). The NCDO763 enzyme was found to exist as a homodimer while that from S3 occurred in two forms, homodimer and homotetramer. It is postulated that these molecular variations are responsible for differences observed in relative activities toward AAA substrates. For example, the tetrameric form was more catalytically efficient toward AAA than the dimeric ATase of S3, but all forms of the enzyme are active under cheese-ripening conditions (pH, temperature, salt concentration).

Degradation of AAA by lactobacilli is also initiated by ATase activity, but specific activity for Trp ATase in *Lactococcus lactis* (Gao et al., 1997) was about 10-fold higher than was found for lactobacilli (Gummalla and Broadbent, 1999). The AAA ATase catalyzes the conversion of Trp, Tyr, and Phe to their respective α -keto acids: IPA, p -hydroxyphenylpyruvate (**HPPA**), and phenylpyruvate (**PPA**). The α -keto acids derived from Trp, Tyr, and Phe are unstable and degrade spontaneously to produce indole-3-aldehyde, hydroxybenzaldehyde, and benzaldehyde, respectively (Gao et al., 1997; Gummalla and Broadbent, 1999). Groot et al. (1998) reported *Lb. plantarum* converted Phe to PPA by transamination, and that PPA could be transformed to benzaldehyde by chemical oxidation. Unlike lactococci, lactobacilli incubated under near-cheese-ripening conditions are able to convert the α -keto acids produced by ATase activity into α -hydroxy acids, which have no significant effect on cheese flavor (Yvon

and Rijnen, 2001). In degradation of AAA, for example, IPA, HPPA, and PPA are converted to ILA, ρ -hydroxyphenyl lactic acid (**HPLA**), and phenyl lactic acid (**PLA**), respectively. These reactions are catalyzed by α -hydroxy acid dehydrogenases (**HA-DH**) such as D-2-hydroxyisocaproate dehydrogenase (**D-HicDH**) (Hummel et al., 1985; Lerch et al., 1989). Since α -hydroxy acids are not believed to have any significant role in flavor development (Yvon and Rijnen, 2001), HA-DHs may be useful in controlling off-flavor development via their ability to divert unstable α -keto acids to more stable enzymic products.

The D-HicDH enzymes of several lactobacilli have been characterized and shown to participate in the stereo-specific reduction of straight and branched chain aliphatic, and aromatic α -keto acids (Bernard et al., 1994; Hummel et al., 1988; Hummel et al., 1985; Lerch et al., 1989; Yamazaki and Maeda, 1986). The nomenclature for this enzyme is derived from the name of the substrate with maximal activity, e.g., α -keto isocaproate, but the substrate pool for this NAD^+ dependent enzyme includes PPA, HPPA, IPA, KMBA, methyl valerate, keto isovalerate, and methyl butyrate. 4-Methylthio-2-oxobutyric acid is produced from Met transamination while the latter three substrates are products of BCAA catabolism. As noted earlier, these compounds may be important in the development of desirable cheese flavor. While D-HicDH enzymes, which produce the D-stereoisomer of the hydroxy acid, are common among LAB, L-HicDH activity has only been reported in *Lactobacillus confusus* (Schutte et al., 1984). The optimal pH of HA-DHs ranges from 5.5 to 7 and their optimal temperature is near 50°C; however, Gummalla and Broadbent (1999) showed D-HicDH activity was present in *L. casei* and *L. helveticus* at the pH, temperature, and salt concentration found in ripening cheese.

Flavor Modifications via Manipulating Amino Acid Metabolisms

Since amino acid metabolism is an essential and rate-limiting step in flavor and aroma development, it should be possible to accelerate cheese flavor and aroma formation by manipulation of AAA, BCAA, and Met catabolism in LAB. This hypothesis is supported by the work of Yvon et al. (1998), who showed addition of α -ketoglutarate to St. Paulin cheese curd increased AAA, BCAA, and Met catabolism and intensified cheese aroma. Banks et al. (2001) corroborated this observation in Cheddar cheese, and agreed this method could serve to accelerate ripening and control flavor development. Rijnen et al. (2000) introduced the gene-encoding glutamate dehydrogenase (*gdh*), which produces α -ketoglutarate from Glu from *Peptostreptococcus asaccharolyticus* into *Lactococcus lactis* and showed enhanced conversion of amino acids to aroma compounds in cheese. The influence of α -ketoglutarate on the production of aroma compounds in St. Paulin cheese was confirmed in another study, which also indicated inactivation of the lactococcal gene encoding aromatic ATase inhibited the formation of floral off-flavor notes (Rijnen et al. 1999b).

The objective of this study was to investigate Tyr and Phe catabolism by two important species of dairy *Lactobacillus*, *L. casei* and *L. helveticus*, under simulated cheese-ripening conditions. It was our hypothesis that an improved understanding of Tyr and Phe catabolism in lactobacilli and the potential of HA-DHs in converting unstable α -keto acids to less reactive α -hydroxy acids can together yield new strategies to control the production of aromatic off-flavors in low fat Cheddar cheese. To investigate this hypothesis, the following specific objectives were explored:

1. Elucidate Tyr and Phe catabolic pathway(s) in *L. casei* and *L. helveticus* cultures under cheese-like conditions and identify enzymes that play a key role in the production of off-flavor compounds.
2. Determine the effect of D-hydroxyisocaproate dehydrogenase overexpression by a *L. casei* adjunct on the chemical and sensory attributes of reduced-fat Cheddar cheese.

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CHAPTER III¹TYROSINE AND PHENYLALANINE CATABOLISM BY
LACTOBACILLUS CHEESE FLAVOR ADJUNCTS

ABSTRACT

Bacterial metabolism of Tyr and Phe has been associated with the formation of aromatic compounds that impart barny-utensil and floral off-flavors in cheese. In an effort to identify possible mechanisms for the origin of these compounds in Cheddar cheese, we investigated Tyr and Phe catabolism by *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts under simulated Cheddar cheese-ripening (pH 5.2, 4% NaCl, 15°C, no sugar) conditions. Enzyme assays of cell-free extracts indicated that *L. casei* strains catabolize Tyr and Phe by successive, constitutively expressed, transamination and dehydrogenation reactions. Similar results were obtained with *L. helveticus* strains, except that the dehydrogenase enzymes were induced during incubation under cheese-ripening conditions. Micellar electrokinetic capillary chromatography of supernatants from *L. casei* and *L. helveticus* strains incubated under simulated cheese-ripening conditions confirmed that Tyr and Phe transamination and dehydrogenation pathways were active in both species and also showed these reactions were reversible. Major products of Tyr catabolism were *p*-hydroxyphenyl lactic acid and *p*-hydroxyphenyl acetic acid, while Phe degradation gave rise to phenyl lactic acid, phenyl acetic acid, and benzoic acid. However, some of these products were likely formed by nonenzymatic processes, since spontaneous chemical degradation of the Tyr

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intermediate ρ -hydroxyphenylpyruvic acid produced ρ -hydroxyphenyl acetic acid, ρ -hydroxyphenyl propionic acid, and ρ -hydroxy benzaldehyde, while chemical degradation of the Phe intermediate phenylpyruvic acid gave rise to phenyl acetic acid, benzoic acid, phenethanol, phenyl propionic acid, and benzaldehyde.

INTRODUCTION

During Cheddar cheese maturation, the amino acids produced from casein degradation are catabolized by the microorganisms in cheese into compounds that can have a strong effect on cheese flavor (Aston and Dulley, 1982; Keeney and Day, 1957; Urbach, 1995). While many of these reactions make positive contributions to cheese flavor, compounds that are thought to originate from the catabolism of aromatic amino acids (AAA) have been shown to impart pungent off-flavors (Dunn and Lindsay, 1985; Guthrie, 1993). Specifically, Dunn and Lindsay (1985) showed that the Phe catabolites phenyl acetaldehyde and phenethanol caused floral, rose-like off-flavors, and that the Tyr catabolite ρ -cresol imparted barny or utensil-like off-flavors. Guthrie (1993) confirmed the roles of phenylacetic acid and phenethanol in off-flavor development in Cheddar cheese, and showed that another Phe metabolite, phenyl propionic acid, also contributed to this defect. Although pathways for the production of each of these molecules from Phe or Tyr have been described in some bacteria (Macfarlane and Macfarlane, 1995), mechanisms for their production in cheese have not yet been established.

Microbial degradation of Tyr and Phe may involve several different enzymes including aminotransferases (**ATase**) (EC 2.6.1.5 and EC 2.6.1.58), decarboxylases (**DCOOHase**) (EC 4.1.1.25 and EC 4.1.1.53), and aromatic hydroxy acid

dehydrogenases (EC 1.1.1.222) (Hemme et al., 1982; Hummel et al., 1986; Macfarlane and Macfarlane, 1995; Schormüller, 1968). Gao et al. (1997) showed the starter cultures used in Cheddar cheese, *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*, both catabolized AAA by ATase under conditions found in ripening cheese (pH 5.2, 4% NaCl, 13°C). That study (Gao et al., 1997) also revealed that nonculturable starter bacteria may contribute to amino acid catabolism in the cheese matrix, and the authors agreed with a previous suggestion that metabolic interconversion of AAA metabolites by starter bacteria and certain lactobacilli could lead to the production of off-flavor compounds (Guthrie, 1993). Because catabolism of AAA and AAA metabolites by starter, adjunct, and nonstarter bacteria appears to be an important source of cheese flavors and off-flavors, knowledge of AAA catabolic pathways in these organisms should reveal useful strategies to control production of the AAA-derived compounds that impart off-flavors to cheese (aromatic off-flavors). Evidence to support the latter hypothesis was recently provided by Rijnen et al. (1999), who reported that starter bacteria that had been genetically engineered to lack the lactococcal aromatic aminotransferase produced St. Paulin-type cheese that had less intense floral notes than cheese made with the wild-type parent strain. While this is an encouraging step toward the control of cheese off-flavor development, the works of Guthrie (1993) and Gao et al. (1997) suggest that full realization of this goal will require a more comprehensive understanding of AAA catabolism by some of the other bacteria that occur in ripened cheese.

Our group is interested in the contribution of lactobacilli to AAA catabolism and cheese off-flavor development because species such as *Lactobacillus helveticus* and *Lactobacillus casei* are widely used as starters or flavor adjuncts, and members of this

genus also dominate populations of nonstarter (adventitious) bacteria in virtually all ripened cheese varieties (El Soda, 1993; Fox et al., 1993; Laleye et al., 1990; Lee et al., 1990). There is little question that lactobacilli can have very positive effects on cheese flavor intensity (El Soda, 1993; Laleye et al., 1990; Lee et al., 1990), but Guthrie (1993) also found that certain lactobacilli, and particularly some strains of *L. casei*, were associated with unclean flavor development in Cheddar cheese. Moreover, AAA metabolites that are known to produce strong off-flavor notes (e.g., *p*-cresol, indole, and skatole) have been recovered from the aroma fractions of *L. helveticus* cultures and from unclean-flavored cheese made with *Lactobacillus* adjuncts (Guthrie, 1993; Kowaleska et al., 1985). As a whole, these data indicate that knowledge of AAA catabolic pathways in adjunct or nonstarter lactobacilli may be vital to industry efforts to control production of aromatic off-flavor compounds in Cheddar and other semi-hard aged cheeses.

Current understanding of the molecular genetics of AAA degradation in dairy lactobacilli is not as advanced as it is for *Lactococcus lactis*, but biochemical data indicate that AAA catabolism in both genera is almost always initiated by an aminotransferase (Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999). For example, Groot et al. (1998) showed that *Lactobacillus plantarum*, a species that is commonly found among cheese nonstarter bacteria (Peterson and Marshall, 1990), converts Phe to phenylpyruvic acid (PPA) by transamination, and that the PPA produced in this reaction may degrade spontaneously into benzaldehyde. Our laboratory previously examined Trp catabolism by *L. casei* and *L. helveticus* cheese flavor adjuncts under simulated cheese-ripening conditions (pH 5.2, 4% NaCl, 15°C, no sugar), and found that strains that are able to catabolize Trp did so via successive transamination and

dehydrogenation reactions to produce indole-3-lactic acid (Gummalla and Broadbent, 1999). Like PPA, however, the α -keto acid intermediate formed in this pathway by Trp transamination (indole pyruvic acid) was chemically labile and could degrade spontaneously into other aromatic compounds (Gao et al., 1997; Gummalla and Broadbent, 1999). In this report, we show that *L. casei* and *L. helveticus* cheese flavor adjuncts use a similar pathway for Tyr and Phe catabolism under cheese-ripening conditions.

MATERIALS AND METHODS

Bacterial Strains

L. helveticus LH212 and *L. casei* LC301 and LC202 were obtained from Rhodia, Inc. (Madison, WI). *L. helveticus* CNRZ32 was provided by J. L. Steele at the University of Wisconsin-Madison. The cultures were propagated in APT broth (Difco, Detroit, MI) at 30°C (*L. casei*) or 37°C (*L. helveticus*), stored at 4°C, and maintained by biweekly transfer.

Preparation of Cell-Free Extracts

Individual lactobacilli were initially screened for enzymes involved in Tyr and Phe catabolism by assays of cell-free extracts (CFE) prepared from 10 ml of an overnight APT culture. The bacteria were harvested by centrifugation for 15 min at $4500 \times g$ (4°C), washed twice with 50 mM potassium phosphate buffer (pH 6.5), and suspended in 1 ml of phosphate buffer. The CFE were prepared by sonic disintegration of the individual cell suspensions with a Branson cell disruptor 200 (Danbury, CT) in pulsed mode at 20

kHz for 5 min in an ice bath. Intact bacteria and cell debris were removed by centrifugation at $4500 \times g$ (4°C) and the supernatant was used as the CFE. Total protein was determined using the Pierce BCA protein assay kit (Rockford, IL) with bovine serum albumin as the protein standard.

To collect information on Tyr and Phe catabolism as it might be likely to occur in cheese, CFE were also prepared from cells that had been incubated in an environment designed to simulate some of the conditions found in ripening Cheddar cheese (Gao et al., 1997; Gummalla and Broadbent, 1999). Bacteria for these studies were harvested by centrifugation from an overnight APT culture, washed twice in a carbohydrate-free chemically defined amino acid medium (CDM) (Gummalla and Broadbent, 1999; Jensen and Hammer, 1993) that lacked L-Tyr or Phe, and suspended in 0.1 volumes of CDM. One milliliter of the cell suspension was then transferred into test tubes that contained 9 ml of CDM that lacked carbohydrate, contained 4% (wt/vol) salt, had been adjusted to pH 5.2 with lactic acid, and either contained or lacked 5 mM L-Tyr or L-Phe. Bacteria suspended in the latter medium (henceforth described as near-cheese-ripening conditions; NCR) were incubated at 15°C , then CFE were prepared by sonic disintegration as described above at time 0 (inoculation) and after 5, 10, 15, and 20 d of incubation.

Identification of Enzymes Involved in Tyr and Phe Catabolism

Tyrosine and Phe ATase activities were measured spectrophotometrically as described previously for Trp ATase activity (Gummalla and Broadbent, 1999). The reaction mixture contained 5 mM L-Tyr or L-Phe, 5 mM α -ketoglutarate, 50 μM pyridoxal phosphate, 5 mM sodium arsenate, and 5 mM EDTA in 50 mM sodium

tetraborate (pH 8.5) buffer. The reaction was initiated by the addition of 250 μ l CFE to obtain a total volume of 1 ml, and the mixture was incubated at 30°C for 30 min. The production of *p*-hydroxyphenylpyruvic acid (**HPPA**) from Tyr or of PPA from Phe was measured by the increase in solution absorbance at 305 nm (A_{305}) or 300 nm (A_{300}), respectively. Specific activities for Tyr and Phe ATase were expressed as μ moles HPPA or PPA produced per mg protein per min. Control reactions without substrate, without CFE, and without substrate and CFE were included in this and all other enzyme assays.

After transamination, HPPA or PPA may be reduced to *p*-hydroxyphenyl lactic acid (**HPLA**) or phenyl lactic acid (**PLA**), respectively, by *p*-hydroxyphenyl lactic acid dehydrogenase (**HPLDHase**) or phenyl lactic acid dehydrogenase (**PLDHase**) (Hummel et al., 1985; Hummel et al., 1986). The CFE were assayed for each of these activities by the spectrophotometric method of Hummel et al. (1986), in which the decrease in reduced NAD^+ (**NADH**) is measured at A_{340} . The reaction mixture contained 250 μ l CFE, 50 mM sodium phosphate (pH 6.5), 5.0 mM HPPA or PPA, and 0.3 mM NADH in a total volume of 1 ml. The specific activity of HPLDHase and PLDHase was reported as μ moles NADH consumed per mg protein per min.

Tyrosine or Phe DCOOHases catalyze the conversion of Tyr to tyramine or Phe to phenethylamine, respectively. The presence of Tyr or Phe DCOOHase activity in CFE was investigated using a 1-ml reaction mixture that contained 5.0 mM L-Tyr or L-Phe, 1 mM pyridoxal phosphate, 250 mM $\text{NH}_4\text{OH-NH}_4\text{Cl}$ (pH 9.0), and 250 μ l CFE (Nakazawa et al., 1977). The mixture was incubated at 30°C for 30 min, terminated by the addition of 0.5 ml trichloroacetic acid, then qualitatively assayed for tyramine or phenethylamine

by micellar electrokinetic capillary chromatography (MECC) with pure standards (Strickland et al., 1996).

Finally, specific activities for Tyr ATase and HPLDHase or Phe ATase and PLDHase were also measured in CFE prepared from cells incubated under NCR for 0, 5, 10, 15, and 20 d as described above. The specific activity values presented for all enzyme assays represent the mean obtained from duplicate experiments replicated on two separate days. The effect of substrate (Tyr versus Phe) and time (d 0 versus 20 under NCR) on enzyme-specific activities was evaluated by statistical t-test comparisons ($\alpha = 0.05$) between means using Microsoft Excel software (Redmond, WA).

Identification of Tyr and Phe Catabolites in Culture Supernatants

Micellar electrokinetic capillary chromatography was also used to identify Tyr and Phe catabolites in the supernatant from cells incubated up to 6 wk under NCR. Lactobacilli for these experiments were prepared from 500 ml culture grown overnight in APT broth. The cells were harvested by centrifugation at $4500 \times g$ (4°C), washed twice in CDM that either lacked L- Tyr or L-Phe, then incubated under NCR in 50 ml of CDM that did or did not contain 5 mM L-Tyr or Phe or one of the following Tyr or Phe metabolites: HPPA, PPA, HPLA, PLA, ρ -hydroxyphenyl acetic acid (HPAA), phenyl acetic acid (PAA), ρ -hydroxyphenyl propionic acid, phenyl propionic acid, ρ -cresol, or phenethanol. Bacterial and control suspensions were incubated under NCR then collected for MECC at time 0 (inoculation) and at weekly intervals thereafter. Sample pH was recorded and viable cell counts determined by plating on APT agar with anaerobic incubation for 48 h. The samples were centrifuged to remove cells and the

supernatants were passed through a Corning (Palo Alto, CA) 0.20 μm cellulose acetate syringe mounted filter, diafiltered through a Filtron (Northborough, MA) 1 K cut-off Microsep concentrator, then diluted 1:5 in a 50 mM sodium tetraborate buffer immediately prior to injection. The MECC was performed in 100 mM SDS - 100 mM sodium tetraborate run buffer as described by Strickland et al. (1996) using a Beckman Instruments P/ACE 2000 (Fullerton, CA) automated capillary electrophoresis system with System Gold software (version 7.11). Peaks that contained Tyr or Phe catabolites were identified by comparisons between the electropherograms obtained from supernatants of cells incubated with and without 5 mM L-Tyr or L-Phe or a Tyr or Phe metabolite. The compound present in each of these peaks was subsequently identified by coinjection with pure standards (Sigma-Aldrich, St. Louis, MO) and by a correlation (r) greater than 0.9 between the absorption spectra from an unknown compound and a known standard. Uninoculated control tubes that contained CDM with 5 mM of L-Tyr or L-Phe or individual Tyr or Phe catabolites were also included in each experiment to identify any compounds that were formed by nonenzymatic degradation (Gummalla and Broadbent, 1999).

RESULTS

Enzymes Involved in Tyr and Phe Degradation in *Lactobacilli*

Tyrosine and Phe ATase activities were detected in CFE collected from all four *Lactobacillus* adjuncts after overnight incubation in APT broth. The specific activity of Tyr ATase was 1.0×10^{-3} $\mu\text{moles/mg protein/min}$ in both strains of *L. helveticus*, and

ranged from 1.4 to 1.7×10^{-3} $\mu\text{moles/mg protein/min}$ in *L. casei* strains. The specific activity of Phe ATase was significantly higher ($P < 0.05$) than L-Tyr ATase activity in all strains, and ranged from 3.5 to 4.1×10^{-3} $\mu\text{moles/mg protein/min}$ in *L. casei*, and 6.0 to 8.0×10^{-3} $\mu\text{moles/mg protein/min}$ in *L. helveticus*. As shown in Table 1, Phe ATase activity was also significantly ($P < 0.05$) higher than Tyr ATase activity when cells were incubated under NCR. The effect of time on Tyr and Phe ATase enzyme activities varied among strains. Incubation for 20 d under NCR did not affect Phe ATase activity in *L. helveticus* LH212 or Tyr ATase activity in *L. casei* LC202 and *L. helveticus* CNRZ32, but the specific activities of these enzymes in the other lactobacilli showed a small but statistically significant ($P < 0.05$) decrease over that period of time.

Cell-free extracts of *L. casei* strains collected after overnight incubation in APT broth also exhibited HPLDHase (8.4 to 11.9×10^{-1} $\mu\text{moles/mg protein/min}$) and PLDHase (6.2 to 7.0×10^{-1} $\mu\text{moles/mg protein/min}$) activities, but neither activity was detected in CFE from *L. helveticus* LH212 or CNRZ32. When *L. casei* strains were incubated under NCR, HPLDHase activity ranged from 5.9 to 12.5×10^{-1} $\mu\text{moles/mg protein/min}$ (Table 2), while PLDHase activity was 14.4 to 19.9×10^{-1} $\mu\text{moles/mg protein/min}$ (Table 2). Interestingly, HPLDHase activity in these cells showed a significant ($P < 0.05$) decrease by d 20, but PLDHase activity increased significantly ($P < 0.05$) over the same period.

Although no HPLDHase or PLDHase activity was detected in CFE from *L. helveticus* cells incubated for 0 or 5 d under NCR, specific activities ranging from 1.1 to 2.4×10^{-1} for HPLDHase and from 1.1 to 4.4×10^{-1} $\mu\text{moles/mg protein/min}$ for

Table1. Specific activity of tyrosine and phenylalanine aminotransferase (ATase) in cell-free extracts from lactobacilli incubated under near-cheese-ripening conditions.¹

Incubation time (d)	<i>Lactobacillus casei</i>				<i>Lactobacillus helveticus</i>			
	LC301		LC202		CNRZ32		LH212	
	Tyr ATase	Phe ATase	Tyr ATase	Phe ATase	Tyr ATase	Phe ATase	Tyr ATase	Phe ATase
0	1.4 ± 0.1	4.8 ± 0.1	1.2 ± 0.0	5.7 ± 0.5	1.3 ± 0.0	5.4 ± 0.0	1.6 ± 0.1	5.0 ± 0.2
5	1.2 ± 0.1	5.2 ± 0.3	1.1 ± 0.2	5.5 ± 0.1	0.9 ± 0.1	6.9 ± 0.0	1.1 ± 0.1	5.5 ± 0.4
10	1.1 ± 0.1	5.4 ± 0.4	1.4 ± 0.1	6.3 ± 0.0	1.0 ± 0.0	6.4 ± 0.2	1.3 ± 0.1	5.8 ± 0.4
15	1.4 ± 0.0	5.6 ± 0.2	1.4 ± 0.1	5.0 ± 0.4	1.1 ± 0.0	5.9 ± 0.3	1.4 ± 0.1	5.2 ± 0.2
20	1.0 ± 0.0	4.6 ± 0.2	1.0 ± 0.1	4.0 ± 0.1	1.2 ± 0.0	4.7 ± 0.1	1.3 ± 0.1	4.9 ± 0.4

¹Chemically defined medium with no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, with 5 mM L-Tyr or L-Phe. Cells were incubated at 15°C. Specific activity values represent $\mu\text{moles } p\text{-hydroxyphenylpyruvic acid or phenylpyruvic acid produced per milligram protein per minute} \times 10^{-3}$ (\pm SE). Values represent the mean from duplicate experiments replicated on two separate days.

Table 2. Specific activity of *p*-hydroxyphenyl lactic acid dehydrogenase (HPLDHase) and phenyllactic acid dehydrogenase (PLDHase) in cell-free extracts from lactobacilli incubated under near-cheese-ripening conditions.¹

Incubation time (d)	<i>Lactobacillus casei</i>				<i>Lactobacillus helveticus</i>			
	LC301		LC202		CNRZ32		LH212	
	HPLDHase	PLDHase	HPLDHase	PLDHase	HPLDHase	PLDHase	HPLDHase	PLDHase
0	12.5 ± 0.3	14.4 ± 1.1	11.8 ± 1.3	11.5 ± 1.3	ND ²	ND ²	ND ²	ND ²
5	8.0 ± 0.1	17.2 ± 1.0	10.8 ± 2.6	18.2 ± 0.5	ND ²	ND ²	ND ²	ND ²
10	7.4 ± 0.2	18.1 ± 0.3	8.6 ± 1.3	17.1 ± 0.4	0.7 ± 0.1	1.0 ± 0.1	0.2 ± 0.1	0.8 ± 0.1
15	6.2 ± 0.4	19.9 ± 1.8	7.0 ± 0.2	18.0 ± 0.6	1.5 ± 0.3	1.1 ± 0.1	1.1 ± 0.1	3.4 ± 0.2
20	6.1 ± 0.1	19.0 ± 0.9	5.9 ± 0.5	18.0 ± 0.8	2.4 ± 0.5	4.4 ± 0.2	1.9 ± 0.2	3.0 ± 0.2

¹Chemically defined medium with no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, with 5 mM L-Tyr or L-Phe. Cells were incubated at 15°C. Specific activity values represent $\mu\text{moles NADH consumed per milligram protein per minute} \times 10^{-1}$ (\pm SE).

Values represent the mean from duplicate experiments replicated on two separate days.

²Not detected.

PLDHase were found in cells incubated for 10, 15, and 20 d (Table 2). Moreover, HPLDHase activity in *L. helveticus* LH212 and PLDHase activity in both *L. helveticus* strains increased significantly ($P < 0.05$) from d 10 to 20. Finally, production of tyramine or phenethylamine was not detected from Tyr or Phe DCOOHase assays of CFE from any of the *Lactobacillus* spp. used in this study.

MECC Analysis

When lactobacilli were incubated under NCR, media pH remained relatively constant ($\text{pH } 5.2 \pm 0.5$), but numbers of culturable cells fell from approximately 10^9 to 10^5 or fewer cfu per ml over a 6 wk period (Figures 1-8). As is summarized in Table 3, MECC analysis of culture supernatants showed all four lactobacilli catabolized Tyr under NCR by same pathway; Tyr was converted to HPLA and HPAA (Figures 1-4), and incubations in CDM with HPLA led to the production of HPAA and Tyr. As expected, supernatant from cells incubated in CDM with HPPA contained Tyr, HPLA, and HPAA. Production of *p*-cresol was not detected by any of the *Lactobacillus* strains tested, but other compounds including *p*-hydroxyphenyl propionic acid and *p*-hydroxy benzaldehyde were found in CDM with HPPA. These products were also detected in cell-free control tubes, however, which showed they were formed by nonenzymatic degradation of HPPA. Further catabolism of *p*-hydroxyphenyl acetic acid and *p*-hydroxyphenyl propionic acid by lactobacilli was not detected (Table 3).

Studies of Phe metabolism under NCR yielded similar results (Table 4). All four lactobacilli converted Phe to PLA, PAA, and benzoic acid (**BA**) (Figures 5-8), and incubations in CDM with PLA led to the production of PAA, BA, and Phe. Supernatant

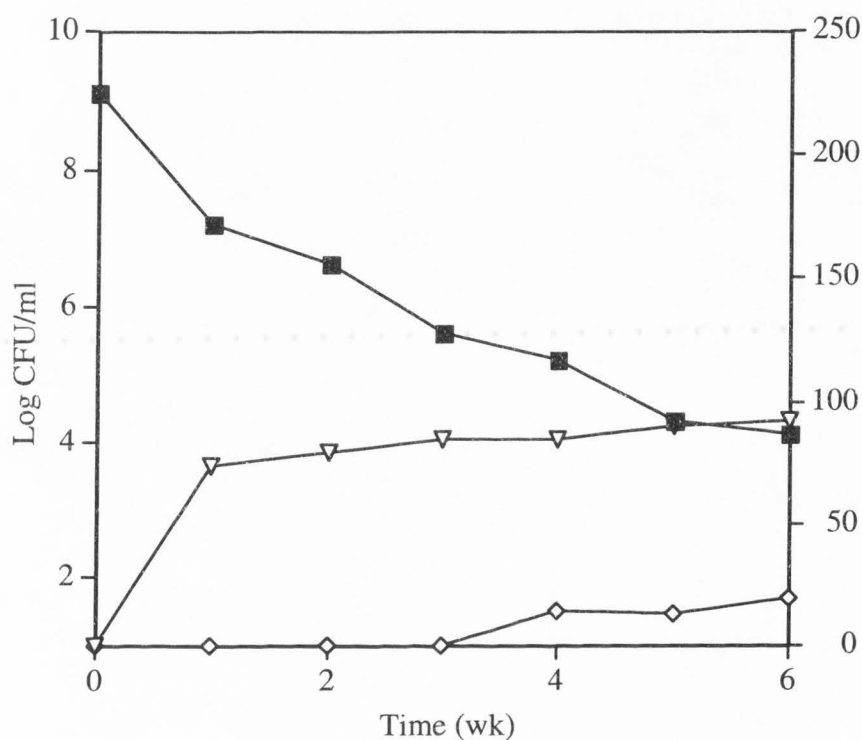


Figure 1. Tyrosine metabolism by *Lactobacillus casei* LC202 incubated under near-cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Tyr. The figures show the number of colony forming units per ml recovered (■) and the concentrations of p-hydroxyphenyl lactic acid (▽) and p-hydroxyphenyl acetic acid (◇) that accumulated in culture supernatant over a 6-wk incubation under NCR.

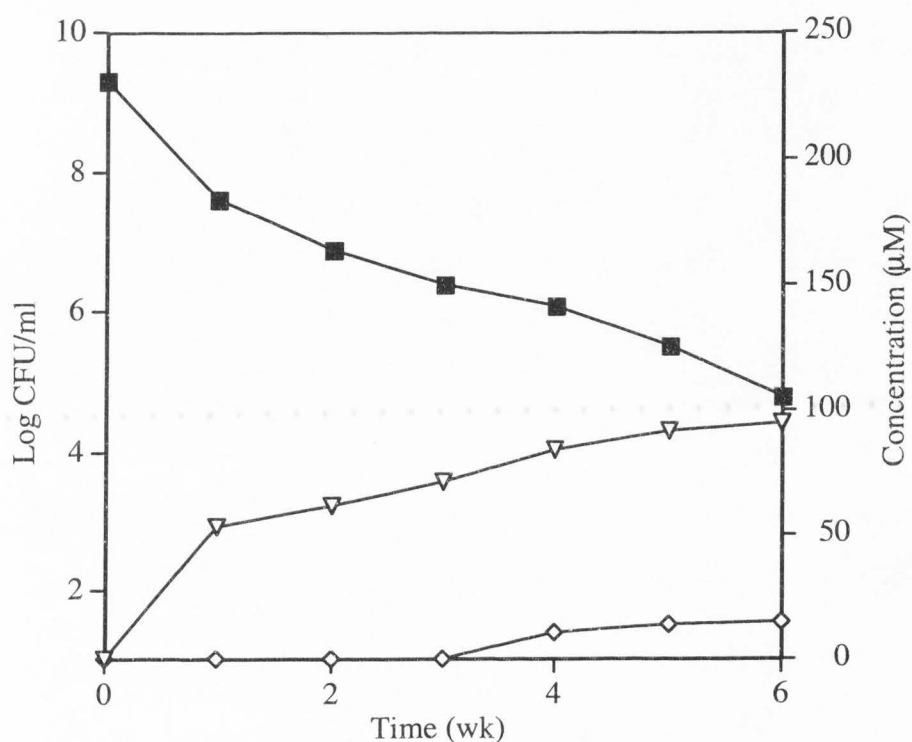


Figure 2. Tyrosine metabolism by *Lactobacillus casei* LC301 incubated under near-cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Tyr. The figures show the number of colony forming units per ml recovered (■) and the concentrations of p-hydroxyphenyl lactic acid (∇) and p-hydroxyphenyl acetic acid (◇) that accumulated in culture supernatant over a 6-wk incubation under NCR.

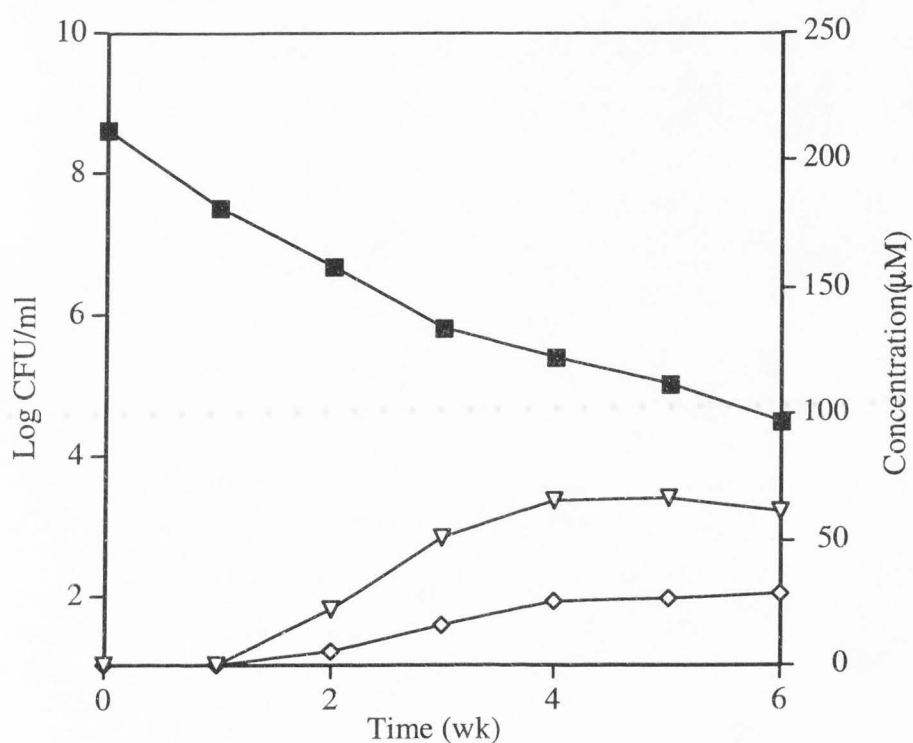


Figure 3. Tyrosine metabolism by *Lactobacillus helveticus* LH212 incubated under near cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Tyr. The figures show the number of colony forming units per ml recovered (■) and the concentrations of p-hydroxyphenyl lactic acid (▽) and p-hydroxyphenyl acetic acid (◇) that accumulated in culture supernatant over a 6-wk incubation under NCR.

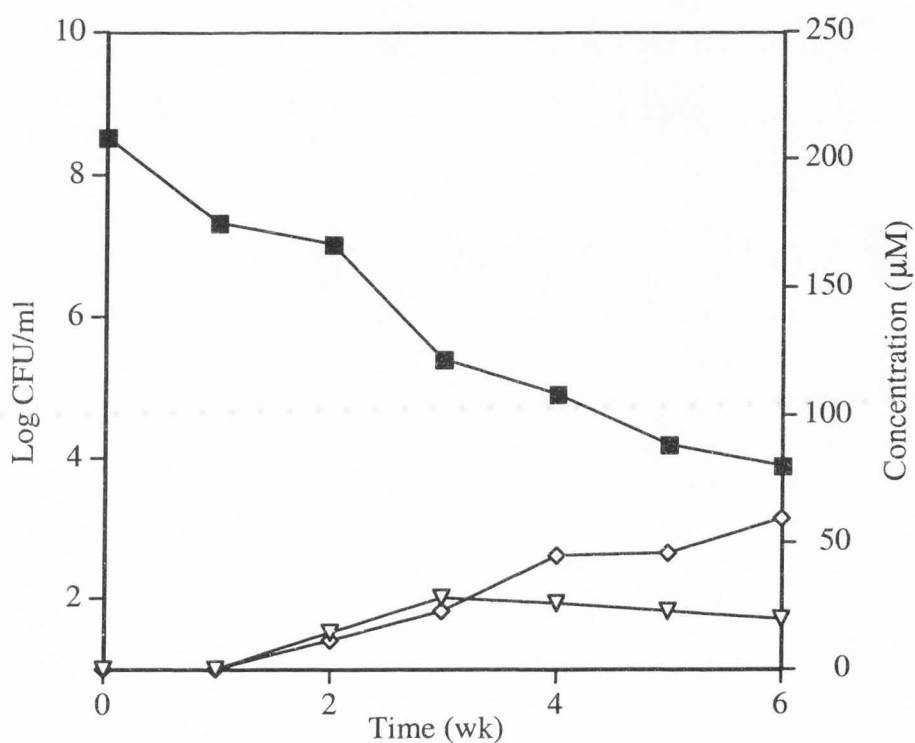


Figure 4. Tyrosine metabolism by *Lactobacillus helveticus* CNRZ32 incubated under near cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Tyr. The figures show the number of colony forming units per ml recovered (■) and the concentrations of p-hydroxyphenyl lactic acid (▽) and p-hydroxyphenyl acetic acid (◇) that accumulated in culture supernatant over a 6-wk incubation under NCR.

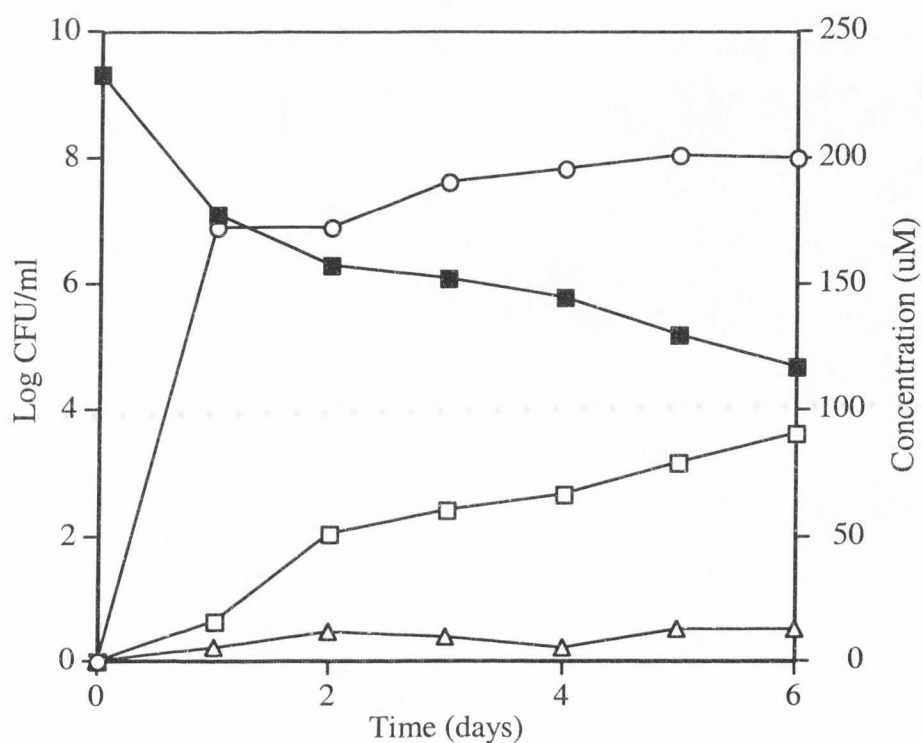


Figure 5. Phenylalanine metabolism by *Lactobacillus casei* LC202 incubated under near cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Phe. The figures show the number of colony forming units per ml recovered (■) and the concentrations of phenyl lactic acid (O), phenyl acetic acid (□), and benzoic acid (Δ) that accumulated in culture supernatant over a 6-wk incubation under NCR.

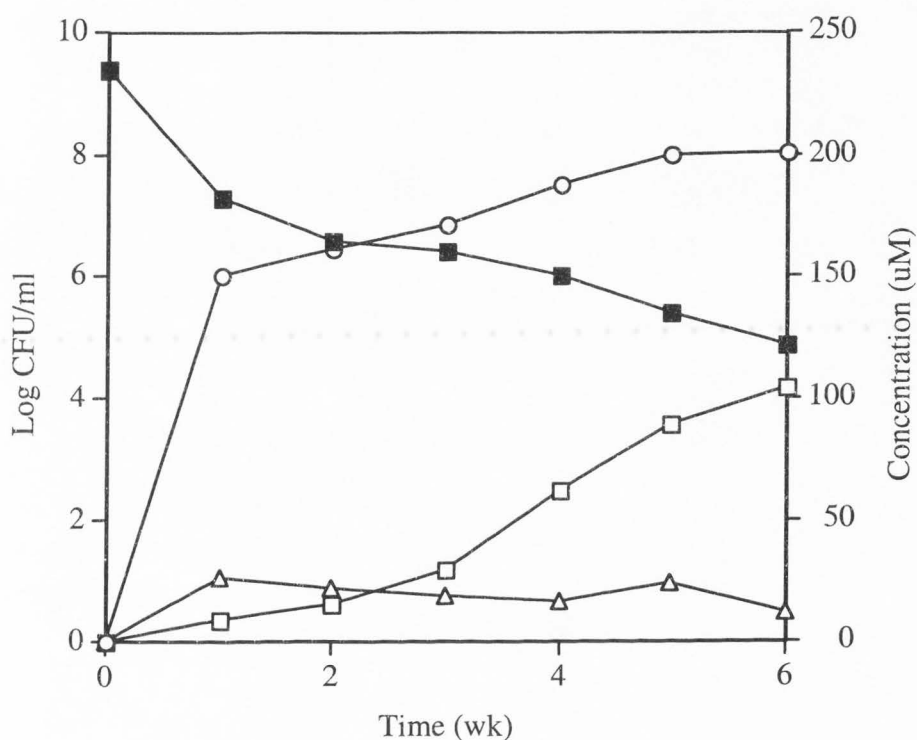


Figure 6. Phenylalanine metabolism by *Lactobacillus casei* LC301 incubated under near-cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Phe. The figures show the number of colony forming units per ml recovered (■) and the concentrations of phenyl lactic acid (O), phenyl acetic acid (□), and benzoic acid (Δ) that accumulated in culture supernatant over a 6-wk incubation under NCR.

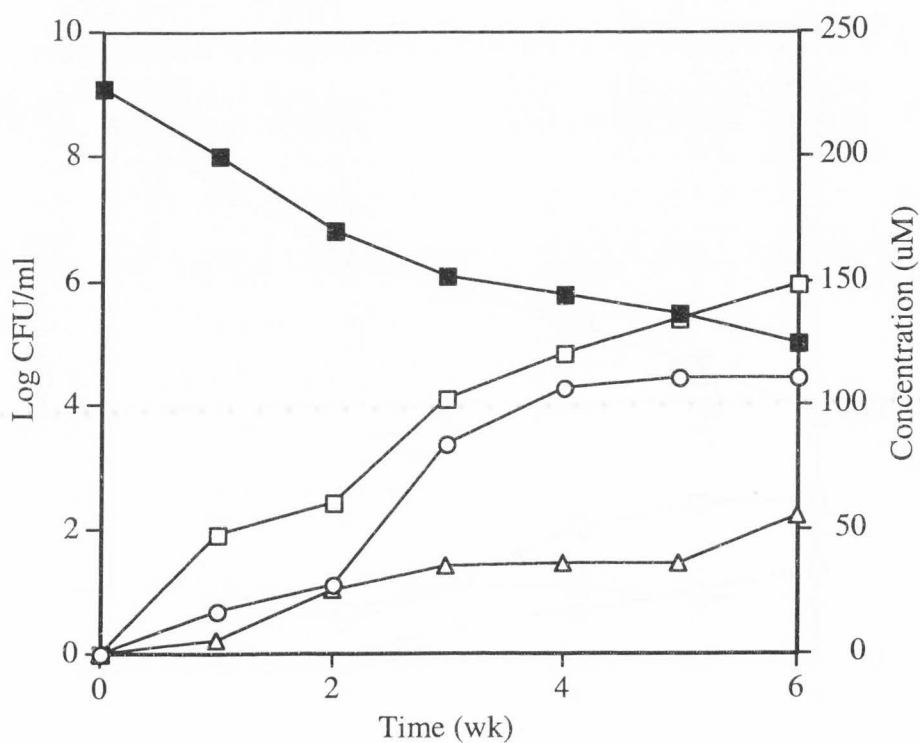


Figure 7. Phenylalanine metabolism by *Lactobacillus helveticus* LH212

incubated under near-cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Phe. The figures show the number of colony forming units per ml recovered (■) and the concentrations of phenyl lactic acid (O), phenyl acetic acid (□), and benzoic acid (Δ) that accumulated in culture supernatant over a 6-wk incubation under NCR.

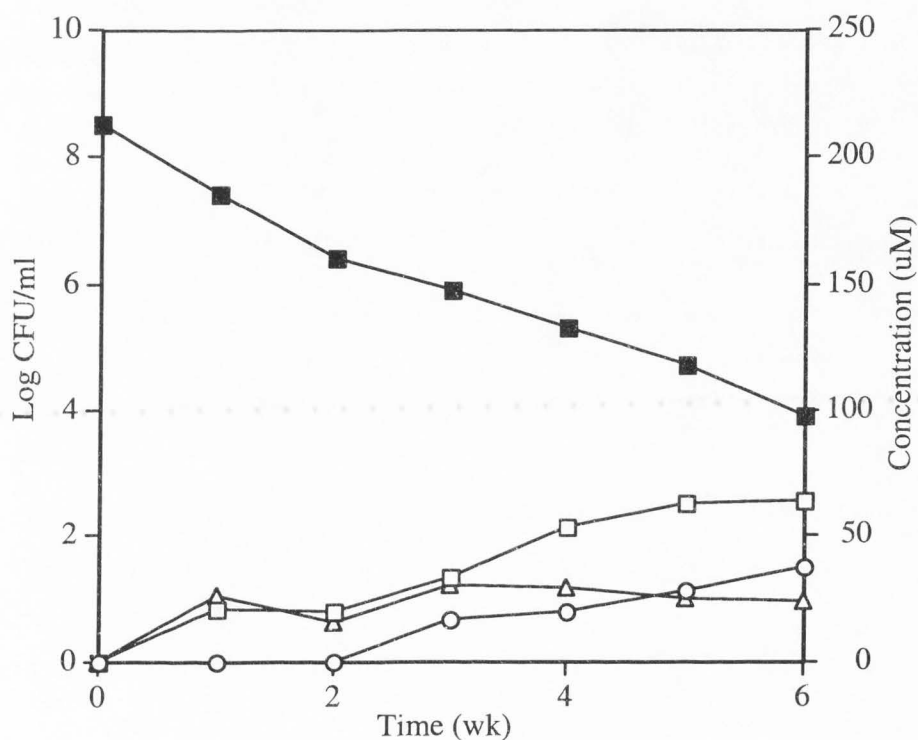


Figure 8. Phenylalanine metabolism by *Lactobacillus helveticus* CNRZ32

incubated under near-cheese-ripening conditions (NCR) in chemically defined medium containing 5 mM L-Phe. The figures show the number of colony forming units per ml recovered (■) and the concentrations of phenyl lactic acid (O), phenyl acetic acid (□), and benzoic acid (Δ) that accumulated in culture supernatant over a 6-wk incubation under NCR.

Table 3. Tyrosine metabolites detected by micellar electrokinetic capillary chromatography of culture supernatant from *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts.¹

Incubation medium	Metabolites detected ²					
	Tyr	HPLA	HPAA	HBAla	HPProA	p-cresol
CDM	-	-	-	-	-	-
CDM + 5 mM Tyr	+	+	+	-	-	-
CDM + 5 mM HPPA	+	+	+ ³	+ ³	+ ³	-
CDM + 5 mM HPLA	+	+	+	-	-	-
CDM + 5 mM HPAA	-	-	+	-	-	-
CDM + 5 mM p-cresol	-	-	-	-	-	+
CDM + 5 mM HPProA	-	-	-	-	+	-

¹Cells were incubated in chemically defined, near-cheese-ripening (no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, 15°C) medium (CDM) spiked with Tyr or a Tyr metabolite.

²Abbreviations: HPPA, p-hydroxyphenylpyruvic acid; HPLA, p-hydroxyphenyl lactic acid; HPAA, p-hydroxyphenyl acetic acid; HBAla, hydroxy benzaldehyde; HPProA, p-hydroxyphenyl propionic acid. Symbols identify compounds that were (+) or were not (-) detected.

³Cell-free controls showed this compound is produced by spontaneous chemical degradation of HPPA.

Table 4. Phenylalanine metabolites detected by micellar electrokinetic capillary chromatography of culture supernatant from *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts.¹

Incubation medium	Metabolites detected ²						
	Phe	PLA	PAA	BA	Phen	BAlD	PProA
CDM	-	-	-	-	-	-	-
CDM + 5 mM Phe	+	+	+	+	-	-	-
CDM + 5 mM PPA	+	+	+ ³	+ ³	+ ³	+ ³	+ ³
CDM + 5 mM PLA	+	+	+	+	-	-	-
CDM + 5 mM PAA	-	-	+	-	-	-	-
CDM + 5 mM Phen	-	-	-	-	+	-	-
CDM + 5 mM PProA	-	-	-	-	-	-	+

¹Cells were incubated in chemically defined, near-cheese-ripening (no carbohydrate, pH 5.2, 4% (wt/vol) NaCl, 15°C) medium (CDM) spiked with Phe or a Phe metabolite.

²Abbreviations: PPA, phenylpyruvic acid; PLA, phenyl lactic acid; PAA, phenyl acetic acid; BAlD, benzaldehyde; BA, benzoic acid; PProA, phenyl propionic acid; Phen, phenethanol. Symbols identify compounds that were (+) or were not (-) detected.

³Cell-free controls showed this compound is produced by spontaneous chemical degradation of PPA.

from cells incubated in CDM with PPA yielded Phe, PLA, PAA, and BA.

Phenethanol, phenyl propionic acid, and benzaldehyde were also detected in CDM with PPA, but these compounds also appeared in cell-free control tubes, which confirmed they were formed by spontaneous chemical degradation of PPA (Gao et al., 1997; Groot et al., 1998; Urbach, 1995). Further degradation of phenyl acetic acid, phenethanol, or phenyl propionic acid was not detected.

Although pathways for Tyr and Phe catabolism under NCR were similar in all four lactobacilli, levels of HPLA, HPAA, PLA, and PAA that accumulated in CDM spiked with Tyr or Phe differed between *L. casei* and *L. helveticus* adjuncts, and also between strains of *L. helveticus*. In CDM spiked with Tyr, for example, HPLA was detected 1 wk earlier in supernatant from *L. casei* versus *L. helveticus* adjuncts, and the former species produced about 2- to 4-fold more HPLA than the latter species (Figures 1-4). In contrast, HPAA was detected in supernatant from *L. helveticus* strains 2 wk before it was found in supernatant from *L. casei* LC301 or LC202. However, the concentration of HPAA in culture supernatants after 6 wk incubation under NCR was similar for all strains except *L. helveticus* CNRZ32, which produced 2- to 3-fold more HPAA than any of the other lactobacilli. Among *L. helveticus* adjuncts, strain LH212 produced more than twice the level of HPLA detected with CNRZ32, while the opposite was true for HPAA (Figure 3 versus 4). *L. helveticus* CNRZ32 was also the only strain that produced more HPAA than HPLA.

As is shown in Figures (5-8), *L. casei* strains incubated under NCR in CDM spiked with Phe produced PLA more rapidly than *L. helveticus* strains, and the concentration of PLA in supernatant from the former species remained several fold

higher than that detected in comparable samples from *L. helveticus* strains. Another difference between these two species was that supernatant from *L. casei* always contained more PLA than PAA, while the opposite was true of *L. helveticus*. Supernatant from *L. helveticus* adjuncts also contained more BA than was found in samples from either strain of *L. casei* (Figures 5-8).

Concentrations of Phe metabolites in supernatant from *L. casei* adjuncts incubated 6 wk under NCR were relatively similar (Figure 5 versus 6), but substantive differences were noted between *L. helveticus* strains (Figure 7 versus 8). For example, PLA production by *L. helveticus* CNRZ32 was not detected until the third week of incubation under NCR, and the concentration of this and all other Phe metabolites in supernatant from cells incubated 6 wk under NCR was less than half that found for *L. helveticus* LH212 (or for either *L. casei* adjunct) (Figures 5-8).

Finally, the higher Phe versus Tyr ATase activity noted in CFE from cells incubated under NCR was also reflected in the cumulative concentrations of Phe and Tyr metabolites that accumulated in supernatant from all lactobacilli except CNRZ32. As is shown in the above figures, levels of PLA plus PAA and BA in supernatant from LC202, LC301, and LH212 cells that had been incubated under NCR in CDM with Phe were about 3-fold higher than the concentration of HPLA plus HPAA in supernatant from cells incubated in CDM with Tyr.

DISCUSSION

In Cheddar cheese, Phe and Tyr metabolites such as PAA, phenethanol, phenylpropionic acid, and *p*-cresol have been shown to impart potent floral, barny, or

utensil off-flavors (Dunn and Lindsay, 1985; Guthrie, 1993). However, because pathways for the production of these compounds in cheese have not yet been established, the individual contributions of starter, adjunct, and nonstarter bacteria to cheese off-flavor development remain unclear. To investigate the contribution of lactobacilli to off-flavor production in Cheddar cheese, our laboratory has analyzed AAA catabolism by *L. casei* and *L. helveticus* cheese flavor adjuncts (Gummalla and Broadbent, 1999). In this report, we describe pathways for Tyr and Phe catabolism by these two species in an environment that simulated some of the conditions found in ripening Cheddar cheese (e.g., no carbohydrate, suboptimal growth temperature, pH 5.2, and 4% NaCl).

Enzyme assays of CFE from *L. casei* LC202 and LC301 indicated both bacteria were likely to catabolize Tyr and Phe through successive transamination and dehydrogenation reactions. Strong evidence that this pathway is the primary route for Tyr and Phe catabolism in cheese was obtained by MECC analysis of culture supernatant from cells incubated under NCR in CDM with Tyr (Table 3) or Phe (Table 4). The finding that Tyr and Phe catabolism by these strains was initiated by an ATase is consistent with our previous data for Trp catabolism by these strains and with other reports of AAA catabolism in dairy lactic acid bacteria (Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999; Hemme et al., 1982). Data presented in Tables 3 and 4 also indicated that Tyr ATase, Phe ATase, HPLDHase, and PLDHase have reversible, anabolic activity because Tyr or Phe were produced from each of their respective products.

The specific activity of Phe or Tyr ATase in CFE from lactobacilli incubated under NCR for up to 20 d was relatively similar in both *Lactobacillus* species (Table 1),

but HPLDHase and PLDHase expression in these bacteria differed both temporally and quantitatively. As is shown in Table 2, the latter enzyme activities were constitutively expressed in CFE from *L. casei*, but neither was initially detected in strains of *L. helveticus*. However, HPLDHase and PLDHase activities were detected in CFE from *L. helveticus* LH212 and CNRZ32 cells that had been incubated more than 5 d under NCR (Table 2). These observations indicate that *L. helveticus* HPLDHase and PLDHase are inducible under NCR, but it is not known if enzyme induction was in response to carbohydrate starvation, low temperature (15 °C), salt (4 % NaCl), pH (5.2), or a combination of two or more of these factors. Furthermore, these specific activities from *L. helveticus* remained at least 6-fold lower than those found in CFE from *L. casei* at comparable sampling times. The differences noted in HPLDHase and PLDHase activities from *L. casei* versus *L. helveticus* strains suggest that the former species likely plays a more prominent role in dehydration of aromatic α -keto acids in the cheese matrix. This distinction may be significant to cheese flavor development because: 1) the α -keto acids formed by ATase reactions are chemically labile; 2) products formed by spontaneous degradation of these acids (e.g., PAA, phenethanol, and phenylpropionic acid) have been shown to impart off-flavors to Cheddar cheese; and 3) once formed, these compounds do not appear to undergo further catabolism by *L. casei* or *L. helveticus* (Tables 3 and 4). As a result, cheese made with *L. helveticus* or other bacteria with low-level HPLDHase and PLDHase activities is likely to contain higher concentrations of aromatic compounds formed spontaneously from the breakdown of HPPA and PPA than cheese made with microorganisms that more actively convert aromatic α -keto acids into aromatic α -hydroxy acids. This hypothesis is supported by

data in Figures 1 and 2, which show levels of HPAA, PAA, and BA were almost always higher in supernatant from *L. helveticus* versus *L. casei* adjuncts incubated under NCR, while the opposite was true for HPLA and PLA.

Finally, some cheese-related bacteria are reported to possess AAA DCOOHase activity, and aromatic amines have been isolated from ripening cheese (Hemme et al., 1982; Moreno-Aribas et al., 2000; Schormüller, 1968). Data collected from cells incubated under NCR in our laboratory, however, indicate that the *L. casei* LC202 and LC301 and *L. helveticus* LH212 and CNRZ32 do not contribute to the production of tyramine, phenethylamine, or tryptamine in Cheddar cheese (Gummalla and Broadbent, 1999).

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CHAPTER IV¹

OVEREXPRESSION OF *LACTOBACILLUS CASEI* D-HYDROXYISOCAPROIC
ACID DEHYDROGENASE: EFFECTS ON CHEESE VOLATILE AND
SENSORY PROPERTIES

ABSTRACT

Lower fat Cheddar cheeses usually lack typical Cheddar notes and are susceptible to off-flavor development. Metabolism of aromatic amino acids by lactic acid bacteria is an important source of unclean and off-flavor compounds in cheese. Previous work has shown that the α -keto acids produced by the action of lactic aminotransferase enzymes on Trp, Tyr, and Phe are chemically labile and may degrade spontaneously into a variety of off-flavor compounds. However, dairy lactobacilli can convert unstable α -keto acids to more stable α -hydroxy acids via the action of α -keto acid dehydrogenases such as D-hydroxyisocaproic acid dehydrogenase. To further characterize the role of this enzyme in cheese flavor, we examined the effect of D-hydroxyisocaproic acid dehydrogenase overexpression in a *Lactobacillus casei* adjunct on the chemical and sensory properties of cheeses manufactured with and without added α -ketoglutarate. The D-hydroxyisocaproic acid dehydrogenase gene was cloned into the high copy number vector pTRKH2 and transformed into *L. casei* ATCC334. Reduced-fat Cheddar cheeses were then made with *Lactococcus lactis* starter only, starter and *L. casei* ATCC334, and starter plus *L. casei* ATCC334 transformed with pTRKH2: *D-HicDH*; then cheese volatiles were measured at 3 mo using gas chromatography-mass spectrometry. Statistical analyses showed culture

¹Coauthored by S. Gummalla, J. E. Hughes, M. E. Johnson, S. Rankin, M. A. Drake, and J. R. Broadbent.

system significantly affected the concentrations of various ketones, aldehydes, alcohols, esters, sulfur compounds, and benzaldehyde in cheese. Results further indicated that overexpression of D-hydroxyisocaproic acid dehydrogenase suppressed spontaneous degradation of α -keto acids but the net effect appeared to retard cheese flavor development.

INTRODUCTION

Microbial catabolism of amino acids generated from degradation of milk proteins during cheese maturation is an essential and rate-limiting step in cheese flavor development (Banks et al., 2001; Rijnen et al., 1999a; Smit et al., 2000; Urbach, 1995; Yvon et al., 1997; Yvon et al., 1998). Many of these reactions impact cheese flavor in a beneficial way. For example, breakdown of Met leads to the production of methanethiol, a compound associated with typical Cheddar notes (Christensen and Reineccius, 1995; Urbach, 1993; Weimer et al., 1999). On the other hand, compounds derived from catabolism of aromatic amino acids (AAA) are implicated in the development of off-flavors in cheese. For example, Phe catabolites phenylacetaldehyde and phenethanol cause floral, rose-like off-flavors and the Tyr catabolite *p*-cresol imparts barny, utensil-type off-flavors (Dunn and Lindsay, 1985; Guthrie, 1993). While mechanisms for production of these and other off-flavor compounds in cheese have not been conclusively established, research has demonstrated lactococci and lactobacilli produce aminotransferases (ATase) that degrades AAA under cheese-ripening conditions (pH 5.2, 4% NaCl, 13°C), and the aromatic α -keto acids produced by these reactions can degrade into phenylacetaldehyde, phenethanol, and other aroma compounds (Gao et al., 1997;

Groot et al., 1998; Gummalla and Broadbent, 1999; Gummalla and Broadbent, 2001).

Unlike lactococci, however, *L. casei* and *L. helveticus* cheese flavor adjuncts incubated under cheese-ripening conditions produce α -hydroxy acid dehydrogenases (HA-DH) (Hummel et al., 1985) such as D-hydroxyisocaproic acid dehydrogenase (D-HicDH) (Lerch et al., 1989) that convert α -keto acids to α -hydroxy acids. Since α -hydroxy acids do not make a significant contribution to flavor development (Yvon and Rijnen, 2001), this class of enzymes may be useful in controlling off-flavor development via their ability to divert the spontaneous degradations of α -keto acids to more stable enzymic products. The D-HicDH enzyme participates in the stereospecific reduction of straight and branched chain aliphatic and aromatic α -keto acids. In addition to α -keto isocaproate, the substrate pool for this NAD⁺ dependent enzyme also includes α -keto acids produced by ATase action on AAA like phenylpyruvic acid (PPA) (from Phe), indole pyruvic acid (IPA) (from Trp) and p-hydroxyphenylpyruvic acid (HPPA) (from Tyr), and α -keto acids produced from Met and branched chain amino acids (BCAA). Since the latter compounds are thought to make important contributions to cheese aroma (Hansen et al., 2001), one potential limitation to D-HicDH overexpression in cheese is that it may suppress development of both undesirable and desirable flavors.

To further characterize the role of this enzyme in cheese flavor, we examined the effect of D-hydroxyisocaproic acid dehydrogenase overexpression in a *Lactobacillus casei* adjunct on the chemical and sensory properties of cheeses manufactured with and without added α -ketoglutarate.

MATERIALS AND METHODS

Chemicals

Amino acids, α -keto acids, pyridoxal 5'-phosphate (PLP), α -ketoglutarate (KG), NADH, erythromycin (Em), lysozyme, diethyl ether, N-undecylactone, and tridecane were obtained from Sigma Chemical Co. (St. Louis, MO.).

Bacterial Strains, Plasmids, and Growth Conditions

Lactococcus lactis D11 was obtained from Rhodia, Inc. (Madison, WI) and *Lactobacillus casei* ATCC334 (Dicks et al., 1996) was obtained from the American Type Culture Collection (Rockville, MD). *Lactobacillus casei* LC202 was obtained from Rhodia, Inc. (Madison, WI) and *Escherichia coli* SURE was obtained from our laboratory culture collection. Frozen stock samples of each culture were stored at -70°C and propagated in Difco APT broth (Detroit, Mich.) at 30°C (*L. lactis* D11 and *L. casei* 202) or 37°C (*L. casei* 334 and *E. coli*). Plasmid pTRKH2 (O'Sullivan and Klaenhammer, 1998) was obtained from T. Klaenhammer of North Carolina State University, Raleigh.

Isogenic Strain Construction

To evaluate the effect of D-HicDH overexpression on cheese chemistry, the *L. casei* LC202 *D-HicDH* gene was isolated by the polymerase chain reaction (PCR) and cloned into the high copy number plasmid pTRKH2. Amplification was performed using 31-mer forward (5'-AAGCACTCGAGATACCGGTGACTTACCATGG-3') and reverse (5'-CGTTATCTGCAGATTGCCGTCTCCTTGTTTCG-3') primers that were

concatenated with *XhoI* and *PstI* linkers, respectively. Template DNA for PCR was isolated as previously described (Broadbent et al., 1998), then amplification of the 1.5-kilobase pair region encoding *D-HicDH* was performed in a Perkin Elmer DNA Thermal Cycler Model 480 programmed for 30 cycles of 94°C for 15 s, 55°C for 60 s, and 72°C for 90 s. The amplicon was cut with *XhoI* and *PstI*, ligated into *XhoI/PstI*-digested pTRKH2, then electroporated into *E. coli* Sure (Promega Corp. Madison, WI). Selection of transformants was performed by spread plating on APT agar (Detriot, MI) that contained 50 µg per ml of Em. Plasmid DNA was isolated by the alkaline lysis method (Sambrook et al., 1989) and the *D-HicDH* insert was confirmed by agarose gel electrophoresis and then sequenced by fluorescent dideoxy chain termination on a Perkin-Elmer Applied Biosystems model 373A automated DNA sequencer. The pTRKH2: *D-HicDH* construct was subsequently designated pBUS1.

Electro-competent *L. casei* 334 were prepared by 2% inoculation of stationary phase cells into 500 ml of MRS (de Man et al., 1960) broth medium. The suspension was incubated at 37°C until it reached an absorbance at A₆₀₀ of 0.8 (Ahrne et al., 1992), then cells were harvested by centrifugation at 5000 × g, washed twice with sterile, distilled water, and suspended in 2.5-ml ice cold, sterile, 30% polyethylene glycol 1450 (Sigma Chemical Co., MO). Three microliters of pBUS1 or pTRKH2 was mixed with 100 µl of cell suspension in a 0.2-cm electroporation cuvette and placed on ice for 3 min. An electric pulse was delivered in a Bio-Rad Gene Pulser (BioRad Laboratories, CA) set to the following parameters: 2.5 kV, 25 µF, and 200 Ω. After electroporation, 0.9 ml of pre-warmed (37°C) MRS broth was added and the cells were incubated at 37°C for 2 h. Selection for transformants was performed by spread plating on MRS agar that contained

5 µg per ml of Em. Transformation of *L. casei* 334 by pTRKH2 (henceforth *L. casei* 334em) and pBUS1 (henceforth *L. casei* BUS) was confirmed by agarose gel electrophoresis of cell lysates prepared by the method of Anderson and McKay (1983).

D-Hydroxyisocaproic Acid Dehydrogenase Assay

The D-HicDH activity in *L. casei* 334em and *L. casei* BUS was measured spectrophotometrically as previously described (Gummalla and Broadent, 2001) with PPA, HPPA, IPA, and 2-ketoisocaproate as substrates. Specific activity was expressed as µmoles of NADH consumed per milligram protein per minute, and the values reported represent the mean from duplicate experiments replicated on two separate days.

Cheese Manufacture

Concentrated frozen cell preparations of *Lactococcus lactis* D11, *L. casei* 334em, and *L. casei* BUS starter were prepared by Rhodia Inc. (Madison, WI). Duplicate vats of 50% reduced-fat Cheddar cheese were then manufactured at the University of Wisconsin-Madison from 550 pounds of skim milk as described previously (Broadbent et al., 1998). Vats were inoculated with one of three culture blends: 1) 1.5% *Lactococcus lactis* D11 bulk starter grown in skim milk (Broadbent et al., 1998); 2) *Lactococcus lactis* D11 plus 25 ml (approx. 1×10^8 CFU) *L. casei* 334em; or 3) *Lactococcus lactis* D11 plus 25 ml (approx. 1×10^8 CFU) *L. casei* BUS. After milling, one half of the curd from each vat was dry salted with 2.75% sodium chloride, while the other half was salted with 2.75% sodium chloride and 2% α-ketoglutarate (KG). After salting, cheeses were hooped into 9-kg blocks, pressed overnight, then vacuum-packaged and ripened at 7°C.

Samples of each cheese (approx 20 g) were collected at monthly intervals for enumeration of starter and nonstarter bacteria as described previously (Broadbent et al., 1998), except that *L. casei* 334 em and BUS variants were enumerated on MRS agar that contained 5 µg per ml erythromycin (Em).

Volatile Analysis by Gas Chromatography-Mass Spectrometry

Volatile analysis was performed by gas chromatography and mass spectrometry (GC-MS) by the method of Colchin et al. (2000). Approximately 100 g of each sample was collected after 3 mo ripening and stored in glass jars at -80°C until needed. Samples for GC-MS were prepared from 10 g of shredded cheese mixed with 40 ml of distilled water. N-undecalactone and tridecane were added at 1 µg per g cheese as internal standards and cheese extracts were purged for 40 min with nitrogen gas in a circulating water bath (35 ± 1°C) at a purge rate of 800 ml per min. Adsorbent traps (ORBO-100, Supelco, Bellefonte, PA) used during sample purge were subsequently eluted with distilled diethyl ether. The first 2 ml of solvent eluate was collected and concentrated under nitrogen to approximately 100 µl for sample injection. Separation of volatile compounds collected from cheese samples was achieved using a Hewlett Packard 6890 (HP, Avondale, PA) gas chromatograph equipped with a 60 m × 0.25 (i.d.) mm capillary StabilWax DA column of film thickness 0.5 µm (Restek, Bellefonte, PA). Chromatography parameters included an initial temperature of 40°C for the first 4 min and increased at a rate of 7°C per min to a final temperature of 220°C. A column flow rate of 1.5 ml per min was maintained following a 2 µl sample injection.

Mass spectrometry (Hewlett Packard 5973 Series, Avondale, PA) of cheese volatiles was performed in electron impact ionization mode with an ionization voltage of 70 eV and mass-scan range between 29 and 400 m/z. Identity of volatile compounds was determined by co-elution with known standards and by comparison of mass spectra with a published database of standard compounds (NIST/EPA/NIH mass spectral library, NIST 98).

Sensory Analysis

Sensory attributes of 3-mo-old cheeses were evaluated in duplicate (2 reps of each treatment x 2 evaluations/rep = 4x/treatment) using 13 sensory flavor attributes for Cheddar cheese (Drake et al., 2000) by judges with more than 150 h training in descriptive sensory analysis of cheese flavor. This analysis was based on a comprehensive descriptive sensory language for flavor and is called the Cheddar cheese lexicon (Drake et al., 2000). Using this technique, flavor descriptors corresponding to specific chemical references were obtained for each cheese flavor profile.

Statistical Analysis

The effect of culture treatment or addition of KG on cheese volatiles and sensory character were evaluated by statistical analysis of variance (ANOVA) with SAS[®] software (SAS Institute, Inc., Cary, NC) using standardized peak areas from GC-MS data. When treatment effects were significant least significant difference pairwise comparison tests were performed to determine which treatment produced the effect. Some data were subject to nonlinear log transformations to normalize data and meet assumptions of homogenous variance.

RESULTS

HA-DH Activity of *L. casei* Isogens

Enzyme assays for D-HicDH activity in *L. casei* 334em and *L. casei* BUS showed HA-DH activity in the latter strain was significantly higher ($P<0.05$) (3- to 6-fold) higher than that of the former, depending on the substrate used (Table 5).

Cheese Composition

The pH of experimental cheeses at hooping ranged from 4.7 to 5.3, and was significantly lower ($P<0.05$) in cheeses with added KG. By d 3, the range of cheese pH was narrower (pH 4.8 to 5.0), but continued to be significantly lower ($P<0.05$) in cheeses with added KG.

As shown in Table 6, adjunct levels in reduced-fat Cheddar cheese made with *L. casei* 334em or BUS exceeded 10^7 CFU per g at 3 d. Numbers of *L. casei* BUS in cheese 2 declined by 2 to 3 orders of magnitude over 3 mo while cheeses made with *L. casei* 334em showed little change (Table 6). Experimental cheeses also contained 10^1 to 10^3 nonstarter lactic acid bacteria per g at d 3, and the levels of these bacteria exceeded 10^6 CFU per g by 3 mo in all cheeses (Table 6). One interesting observation was that Em resistant colonies of lactobacilli recovered from cheeses containing KG were notably larger (> 2 mm) than the pinhead-size colonies (< 1 mm) obtained from cheeses without KG.

Table 5. Specific activities of D-hydroxyisocaproic acid dehydrogenase in cell-free extracts from *Lactobacillus casei* 334em and BUS ¹.

Substrate	<i>Lactobacillus casei</i>	
	334em	BUS
2-Ketoisocaproate	1.2 ± 0.2	6.0 ± 0.2
Indole pyruvic acid	0.3 ± 0.1	1.8 ± 0.2
Phenylpyruvic acid	23.9 ± 1.1	122.6 ± 6.1
<i>p</i> -Hydroxyphenylpyruvic acid	35.1 ± 1.3	113.8 ± 3.2

¹μmoles NADH consumed per milligram protein per minute × 10⁻¹ (±SE)

Effect of α-Ketoglutarate Addition on Cheese Volatiles

Cheese volatile analysis identified a broad range of compounds in each cheese that included ketones, aldehydes, alcohols, fatty acids, and sulfur compounds. The volatile fraction of cheese contained saturated and unsaturated methyl ketones such as 2-heptanone, 2-nonanone, 3-penten-2-one-4-methyl, 8-nonen-2-one, and 2-pentanone-4-methyl. Statistical analysis showed there was a strong positive correlation between ketone accumulation and addition of KG. For example, concentrations of 2-heptanone and 2-nonanone showed a 33% and 31% decrease, respectively, while levels of 3-penten-2-one-4-methyl, 2-undecanone, 2-pentanone-4-methyl, and 3-hydroxy-2-butanone were 36%, 94%, 350%, and 77%, higher, respectively, than corresponding values found in cheeses to which KG was not added (Table 7).

Table 6. Numbers (CFU per g) of viable active starter, adjunct, and nonstarter lactic acid bacteria in experimental Cheddar cheeses during ripening.

Cheese ¹	Starter			Adjunct ²			NSLAB		
	3d	1mo	3 mo	3d	1mo	3 mo	3d	1mo	3 mo
1 A	3.2×10^8	4.7×10^8	5.9×10^5	ND ³	ND ³	ND ³	3.7×10^1	2.0×10^4	3.2×10^7
1 B	1.0×10^8	2.1×10^7	9.5×10^5	ND ³	ND ³	ND ³	1.6×10^1	7.8×10^4	3.1×10^7
2 A	8.4×10^7	2.6×10^8	1.8×10^8	5.8×10^7	3.5×10^7	1.8×10^7	5.7×10^1	3.0×10^4	1.1×10^6
2 B	1.4×10^8	1.6×10^8	1.8×10^8	4.0×10^7	2.0×10^7	6.6×10^6	2.2×10^2	3.7×10^4	2.1×10^6
3 A	2.4×10^8	3.0×10^7	2.6×10^7	7.9×10^6	2.0×10^7	4.3×10^4	7.6×10^3	1.6×10^5	1.0×10^7
3 B	3.0×10^8	1.9×10^7	5.0×10^6	9.7×10^6	3.2×10^7	9.0×10^3	7.7×10^3	7.6×10^4	2.4×10^7

¹Treatments: 1 = *Lactococcus lactis* D11, 2 = *Lactococcus lactis* D11 + *Lactobacillus casei* 334em, 3 = *Lactococcus lactis* D11 + *Lactobacillus casei* BUS, A = with ketoglutarate, B = without ketoglutarate;

²Cells recovered from MRS agar that contained 5 µg per ml Em; ³Not detected

36%, 94%, 350%, and 77%, higher, respectively, than corresponding values found in cheeses to which KG was not added (Table 7).

Acetophenone was also detected in the volatile fraction of all cheeses but no correlation was observed between KG addition and production of this lactone. Interestingly, 2-pentanone was only identified in KG cheese manufactured with *Lactococcus* starter alone. As shown in Table 7, cheese made with KG also contained 17% less nonanal, 73% less decanal, and 47% less hexadecanal than control cheese without KG.

The most abundant primary alcohols detected in this study included 3-methyl-1-butanol and 2-phenethylalcohol, and the most common secondary alcohols included 4-methyl-2-pentanol and 2-heptanol (Table 7). Correlation analysis showed alcohol accumulation was influenced by the addition of KG. For example, concentrations of 2-methyl propanol, 4-methyl-2-pentanol, 1-pentanol, 1-hexanol, 1-octen-3-ol, 1-ethyl hexanol, and 2, 3-butanediol were 290%, 50%, 776%, 156%, 92%, 116%, and 82%, respectively, higher than corresponding values in cheeses to which KG was not added (Table 7). Notably, the accumulation of 1-pentanol was significantly ($P < 0.1$) higher in cheeses with KG (Table 8). Addition of KG did not influence accumulation of 3-methyl-1-butanol, 1-heptanol, 2-heptanol, or phenethanol.

The most abundant volatile fatty acid identified in this work was acetic acid (Table 7), and cheeses made with added KG contained 36% higher levels of this fatty acid. The accumulation of other fatty acids was also influenced by addition of KG. The concentrations of 2-methyl propanoic acid, hexanoic acid, and 3-methyl butanoic acid showed a 97%, 50%, and 65% decrease, respectively, while levels of octanoic acid,

Table 7. Quantification of volatile compounds found in 3-mo-old cheeses.

Compound	Character	Probable source	mean ^A (µg/g)				
			Culture 1 ^B	Culture 2 ^C	Culture 3 ^D	α-KG-A ^E	α-KG-B ^F
2-heptanone	spicy	fatty acids	0.410 ^{ab} (0.306)	0.522 ^a (0.076)	0.187 ^b (0.042)	0.299 (0.130)	0.447 (0.279)
3-hydroxy, 2-butanone	buttery	diacetyl, Leu, Val	0.644 ^a (0.222)	1.406 ^b (0.706)	0.613 ^a (0.287)	1.135 ^a (0.667)	0.641 ^b (0.330)
2-nonanone	fruity	fatty acids	0.387 (0.300)	0.818 (0.517)	0.169 (0.215)	0.373 (0.532)	0.542 (0.342)
4-methyl, 3-penten-2-one	vegetable	fatty acids	1.027 (0.827)	0.849 (0.429)	0.469 (0.050)	0.903 (0.691)	0.662 (0.374)
4-hydroxy, 4-methyl, 2-pentanone		fatty acids	0.134 (0.174)	0.497 (0.877)	0.193 (0.041)	0.450 (0.674)	0.099 (0.112)
2-undecanone	floral	fatty acids	0.006 (0.012)	0.010 (0.019)	0.031 (0.029)	0.021 (0.019)	0.011 (0.026)
8-nonen-2-one		fatty acids	0.012 (0.024)	ND ^G	0.014 (0.028)	ND ^G	0.018 (0.027)
acetophenone	orange	Phe	0.524 (0.854)	1.363 (1.258)	0.064 (0.020)	0.598 (1.225)	0.702 (0.757)
4-methyl-2-pentanol		ketones	0.061 (0.071)	0.318 (0.320)	0.111 (0.053)	0.196 (0.265)	0.131 (0.160)

Table 7. (continued).

Compound	Character	Probable source	Mean ^A µg/g				
			Culture 1 ^B	Culture 2 ^C	Culture 3 ^D	α-KG-A ^E	α-KG-B ^F
3-methyl-1-butanol	fruity	Leu	3.725 (3.438)	3.007 (2.151)	3.242 (2.589)	3.304 (2.679)	3.345 (2.632)
2-methyl-1-propanol	alcohol	Val	0.322 (0.588)	0.062 (0.080)	0.044 (0.051)	0.228 (0.479)	0.058 (0.070)
1-pentanol	alcohol		0.059 (0.078)	0.339 (0.398)	0.047 (0.046)	0.266 ^a (0.328)	0.030 ^b (0.049)
2-heptanol	oily		0.048 (0.059)	0.011 (0.021)	0.017 (0.033)	0.027(0.049)	0.023 (0.035)
1-hexanol	green		0.442 (0.214)	0.604 (0.782)	0.157 (0.093)	0.577 (0.576)	0.225 (0.275)
1-octen-3-ol	mushroom	fatty acids	0.114 ^a (0.113)	0.813 ^b (0.602)	0.049 ^a (0.031)	0.428 (0.507)	0.223 (0.480)
1-heptanol	woody		0.009 (0.018)	0.010 (0.020)	0.024 (0.024)	0.013 (0.016)	0.016 (0.025)
1-ethyl, hexanol	alcohol	fatty acids	0.355 ^a (0.471)	2.378 ^b (1.819)	0.060 ^a (0.043)	1.274 (2.026)	0.588 (0.532)
2, 3-butanediol	creamy	acetoin	0.140 (0.280)	0.779 (1.553)	0.044 (0.088)	0.095 (0.228)	0.547 (1.256)
2-phenethylalcohol	rosy	Phe	3.771 (2.831)	3.217 (1.512)	2.100 (0.217)	3.030 (2.352)	3.028 (1.355)
phenol	medicinal	Tyr	0.941 (0.724)	1.463 (1.425)	0.266 (0.145)	0.543 (0.681)	1.237 (1.170)

Table 7. (continued).

Compound	Character	Probable source	Mean ^A µg/g				
			Culture 1 ^B	Culture 2 ^C	Culture 3 ^D	α-KG-A ^E	α-KG-B ^F
nonanal	grassy	fatty acids	0.441 ^a (0.327)	1.034 ^b (0.453)	0.234 ^a (0.131)	0.515 (0.475)	0.624 (0.491)
decanal	orange		0.097 (0.1540)	0.426 (0.820)	0.030 (0.045)	0.077 (0.123)	0.292 (0.669)
hexadecanal			0.418 (0.480)	0.550 (0.305)	0.216 (0.199)	0.271 (0.229)	0.518 (0.417)
octadecanal			1.452 (2.285)	1.713 (1.986)	0.056 (0.079)	1.233 (1.715)	0.914 (1.943)
acetic acid	sour	citrate	1.469 ^a (0.561)	3.781 ^b (0.946)	0.381 ^c (0.339)	2.168 (1.784)	1.586 (1.494)
butanoic acid	rancid	fatty acids	0.750 (0.905)	1.480 (0.703)	0.189 (0.171)	0.732 (0.924)	1.057 (1.066)
hexanoic acid	pungent	fatty acids	0.261 (0.329)	0.317 (0.186)	0.373 (0.072)	0.341 (0.141)	0.677 (0.953)
2-ethyl hexanoic acid	fruity		0.202 (0.404)	0.172 (0.229)	0.109 (0.129)	0.210 (0.221)	0.112 (0.197)
octanoic acid	waxy	fatty acids	0.128 (0.210)	0.450 (0.327)	0.203 (0.145)	0.354 (0.221)	0.167 (0.281)
3-methyl butanoic acid	sweaty	Leu	0.493 (0.884)	0.356 (0.445)	0.111 (0.145)	0.164 (0.211)	0.476 (0.747)
pentanoic acid	cheesy	fatty acids	0.142 ^a (0.224)	1.264 ^b (1.076)	0.049 ^a (0.035)	0.590 (0.874)	0.380 (0.816)

Table 7. (continued).

Compound	Character	Probable source	Mean ^A µg/g				
			Culture 1 ^B	Culture 2 ^C	Culture 3 ^D	α-KG-A ^E	α-KG-B ^F
Dimethylsulfide	sulfur	Met	0.678 ^{ab} (0.323)	1.357 ^a (0.68)	0.365 ^b (0.107)	0.660 (0.684)	0.940 (0.498)
Benzaldehyde	almond	Phe	0.236 ^a (0.143)	0.935 ^b (0.519)	0.064 ^a (0.020)	0.286 (0.212)	0.537 (0.658)

^AMeans in row with different superscript letters are different ($P < 0.1$) using LSD pair wise mean comparisons. Values in parentheses denote \pm SE (n=3 for culture treatment or n=2 for α -ketoglutarate treatment)

^BAll cheeses manufactured with *Lactococcus lactis* D11 only

^CAll cheeses manufactured with *Lactococcus lactis* D11 + *Lactobacillus casei* 334em

^DAll cheeses manufactured with *Lactococcus lactis* D11 + *Lactobacillus casei* BUS

^EAll cheeses with added α -ketoglutarate

^FAll cheeses without α -ketoglutarate

^GNot detected

pentanoic acid, and 2-ethyl hexanoic acid were 112%, 55%, and 87%, respectively, higher than corresponding values found in cheeses to which KG was not added (Table 7).

The only sulfur compounds detected in cheese samples from this study were dimethylsulfide and dimethylsulfoxide. Levels of dimethylsulfide decreased 29% in cheeses made with added KG, while dimethylsulfoxide was identified only in two cheeses containing KG but manufactured with different culture treatments.

Other important volatiles identified include benzaldehyde, phenol, and *p*-cresol. Levels of benzaldehyde and phenol showed a decrease of 46% and 56%, respectively, in cheese made with added KG. Accumulation of phenol in cheese was the same in cheese manufactured with or without KG and *p*-cresol was identified only in one cheese made with added KG.

Effect of Culture on Cheese Volatiles

Statistical ANOVA showed cheese volatile profile was also significantly affected by culture treatment. Culture treatment significantly affected the levels of 2-heptanone ($P < 0.1$), and 3-hydroxy-2-butanone ($P < 0.05$) in cheese (Table 8). Mean concentrations (ppm) of 2-heptanone were highest in cheese made with starter plus *L. casei* 334em (cheese 2) but relatively equal in cheeses made with *Lactococcus lactis* D11 starter alone (cheese 1) or starter plus *L. casei* BUS (cheese 3) (Table 7). Culture treatment could be correlated with changes in levels of nonanal, hexadecanal, and octadecanal, but not decanal (Table 7). The levels of nonanal in cheese 2 were significantly ($P < 0.05$) higher than in cheeses 1 and 3 (Table 8). The concentration of nonanal in cheese 3 was lower than that accumulated in cheese 1, but this difference was

Table 8. Statistical significance of treatment effects on individual volatile components.

Volatile compounds	Culture	α -Ketoglutarate	Aroma/Flavor ²
2-heptanone	($P < 0.1$)	NS ¹	spicy, musty
3-hydroxy-2-butanone	($P < 0.05$)	($P < 0.1$)	buttery
1-pentanol	NS ¹	($P < 0.1$)	unknown
1-octen-3-ol	($P < 0.05$)	NS ¹	mushroom-like
1-ethyl hexanol	($P < 0.05$)	NS ¹	alcohol, pungent
nonanal	($P < 0.05$)	NS ¹	floral, green
acetic acid	($P < 0.05$)	NS ¹	vinegar, sour
pentanoic acid	($P < 0.1$)	NS ¹	cheesy, putrid
benzaldehyde	($P < 0.05$)	NS ¹	bitter almond
dimethylsulfide	($P < 0.1$)	NS ¹	boiled cabbage

¹Not significant

²From Sable and Cottenceau (1999) and Moio and Addeo (1998)

not statistically significant.

Culture treatment significantly altered the production of 1-ethyl hexanol ($P < 0.05$) and 1-octen-3-ol ($P < 0.05$) in cheese (Table 7). Concentrations of both compounds were significantly higher in cheese 2 than in cheeses 1 and 3 (Tables 7 and 8).

The levels of acetic acid were significantly different among cheeses made with different culture systems; they were significantly ($P < 0.05$) higher in cheese 2 than in cheeses 1 and 3 (Table 8). Additionally, concentration of acetic acid in cheese 3 was significantly ($P < 0.05$) lower than that accumulated in cheese 1 (Table 8).

The accumulation of dimethylsulfide in experimental cheeses and culture treatment were significantly correlated (Table 7). Levels of dimethylsulfide in cheese 2 were significantly ($P < 0.05$) higher than in cheese 3 (Table 8). Culture treatment did not affect accumulation of phenol in cheese but the concentration of benzaldehyde in cheese 2 was significantly higher ($P < 0.05$) than in cheeses 1 and 3 (Table 8).

Effect of α -Ketoglutarate and Culture

System on Sensory Properties

Sensory attributes of cheeses that were evaluated included cooked, whey, milk fat, diacetyl, sulfur, nutty, brothy, barny, acid, sweet, salt, bitter, and umami. No significant differences were observed with cooked, whey, milk fat, diacetyl, acid, sweet, and salt flavors between cheeses made with or without KG (Table 9). However, significant KG effects were observed in responses toward nutty ($P < 0.1$), brothy ($P < 0.05$), barny

Table 9. Statistical significance of treatment effects on sensory attributes.

Sensory quality	Volatile association	Culture system	α -Ketoglutarate
Cooked	Lactones	NS ¹	NS ¹
Whey		NS ¹	NS ¹
Milk fat	3-hydroxy-2-butanone	NS ¹	NS ¹
Diacetyl	3-hydroxy-2-butanone	NS ¹	NS ¹
Sulfur	Dimethylsulfide	NS ¹	($P < 0.05$)
Nutty	benzaldehyde, pyrazines	($P < 0.05$)	($P < 0.1$)
Brothy	Methional	($P < 0.1$)	($P < 0.05$)
Barny	<i>p</i> -cresol	NS ¹	($P < 0.05$)
Acid	Acetic acid	NS ¹	NS ¹
Sweet	2-pentanone	NS ¹	NS ¹
Salty		NS ¹	NS ¹
Bitter		NS ¹	($P < 0.05$)
Umami	Mono sodium glutamate	NS ¹	($P < 0.05$)

¹Not significant

($P < 0.05$), bitter ($P < 0.05$), umami ($P < 0.05$), and sulfur ($P < 0.05$) notes (Table 9).

For example, cheeses made with KG scored significantly lower for sulfur, nutty, brothy, barny, and umami flavors, while bitter flavor was higher in these cheeses in comparison with those lacking KG.

No effect of starter system was observed in cooked, whey, milk fat, diacetyl, acid, sweet, salt, sulfur, barny, bitter, and umami flavors, but significant differences were detected in the intensity of nutty ($P < 0.05$) and brothy ($P < 0.1$) flavors (Table 9). Nutty notes were highest in cheese 1 and were scored significantly lower in cheeses 2 and 3. On the other hand, brothy notes were highest in cheese 3 and were significantly lower in cheeses 1 and 2.

Effect of α -Ketoglutarate \times Culture System

Interaction on Cheese Volatiles and Sensory

Properties

The accumulation of most cheese volatiles was not influenced by the effect of interaction between KG and culture system ($P > 0.1$). The exceptions to this observation were 1-hexanol ($P < 0.1$), 1-pentanol ($P < 0.05$), phenol ($P < 0.05$), and benzaldehyde ($P < 0.05$). Levels of benzaldehyde were significantly higher ($P < 0.05$) in cheese made with the culture combination containing *L.casei* 334em and without KG in comparison with cheeses made with any other culture combination either with or without KG.

Although, accumulation of phenol was not significantly influenced by culture treatment or by the addition of KG, the interaction between these two factors caused significant changes in the levels of phenol in cheeses. For example, levels of phenol

were significantly higher ($P < 0.05$) in cheese made with the culture combination containing *L.casei* 334em and without KG in comparison with cheeses made with any other culture combination either with or without KG.

The levels of 1-pentanol and 1-hexanol were significantly higher in cheeses made with addition of KG and culture combination containing *L.casei* 334em than in cheeses made with any other culture combination either with or without KG.

No KG \times Culture system interaction effects were observed on any sensory flavor attributes except with the perception of sulfur notes. Sulfur notes in cheese with added KG and made with either adjunct (*L.casei* 334em or BUS) was significantly lower ($P < 0.05$) than that in cheeses without KG and manufactured with either starter alone or in combination with *L.casei* 334em. Sulfur notes in cheese made with starter alone (*Lactococcus lactis* D11) and containing KG was significantly lower ($P < 0.05$) than all other combinations of starter and KG used in this study. Additionally, cheese made with the culture combination containing *L.casei* BUS and without KG scored significantly less ($P < 0.05$) sulfur flavors than the other two culture combinations made without KG.

DISCUSSION

Catabolism of AAA by lactococci and lactobacilli in the cheese environment is almost exclusively initiated by transaminase, and the α -keto acids produced from these reactions can degrade spontaneously into a variety of off-flavor compounds (Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999; Gummalla and Broadbent, 2001). Previous research by our group on AAA catabolism by *L. casei* under cheese-like conditions revealed these bacteria produce HA-DHs, such as D-HicDH, that convert α -

keto acids into more stable α -hydroxy acid derivatives (Gummalla and Broadbent, 1999; Gummalla and Broadbent, 2001). Consequently, one potential strategy to avoid off-flavors caused by AAA catabolism may be to enzymatically convert the α -keto acids produced in these reactions into α -hydroxy acids, which have no significant effect on cheese flavor (Yvon and Rijnen, 2001). A potential drawback to this strategy is that HA-DH overexpression may also suppress production of desirable flavor notes formed via Met and BCAA catabolism. To investigate the influence of D-HicDH overexpression on cheese chemistry, we developed an isogenic variant of *L. casei* was constructed and used to manufacture 50% reduced-fat Cheddar cheese made with and without 2% KG.

Because catabolism of AAA and their metabolites by lactococci and lactobacilli appears to be a significant source of unclean flavors, genetic manipulation of AAA metabolism may facilitate industry efforts to controlling off-flavor development in cheese. For example, Rijnen et al. (1999b) showed inactivation of the gene encoding the lactococcal aromatic ATase retarded degradation and formation of floral off-odors in St. Paulin-type cheese.

A large number of ketones, aldehydes, alcohols, fatty acids, esters, and sulfur compounds were identified (Table 7). The majority of ketones detected were methyl ketones, which are produced by enzyme catalyzed oxidative decarboxylation of fatty acids (Adda, 1986). Although the significance of methyl ketones in Cheddar flavor is unknown, their contribution to flavor of surface-ripened cheeses is established (Welsh et al., 1989). High concentrations of methyl ketones in Cheddar can be indicative of mold contamination, but some autooxidative pathways of fat degradation are also reported to contribute to the production of these compounds (Fox and Wallace, 1997). It is unclear

how KG may affect the production of ketones, but Banks et al. (2001) also reported that levels of 3-hydroxy-2-butanone (acetoin) were increased with the addition of KG. 3-hydroxy-2-butanone can be formed by enzymatic condensation of two molecules of acetaldehyde or via diacetyl reduction (Hugenholtz, 1993). Additionally, Yvon et al. (1998) showed addition of KG enhanced the degradation of BCAA. This observation may explain the significant ($P < 0.1$) increase in 3-hydroxy-2-butanone production, since Leu and Val catabolism can lead to production of acetolactate, a precursor metabolite for synthesis of 3-hydroxy-2-butanone and diacetyl. Alternatively, KG can act as an organic external electron acceptor causing a pyruvate surplus in cells and thereby be able to drive the production of diacetyl and 3-hydroxy-2-butanone (Salminen and von Wright, 1993). This effect may be beneficial to cheese flavor, since, 3-hydroxy-2-butanone is associated with typical Cheddar notes (Hugenholtz, 1993). Similar overlaps among bacterial metabolic pathways in cheese may be responsible for the observed influence of KG on production of other ketones.

The detection of methyl ketones is also important to cheese flavor since compounds like 2-pentanone can impart an orange peel-like fruity, sweet flavor (Arora et al., 1995) to the aroma of Cheddar but is more common in mold-ripened cheeses, particularly Camembert (Molimard and Spinnler, 1996). Likewise, 2-heptanone and 2-nonanone both impart musty and fruity aromas (Sable and Cottenceau, 1999), while 2-undecanone bears a citrus-rose odor (Moio and Addeo, 1998). 3-Penten-2-one-4-methyl and 2-pentanone-4-methyl carry pungent and fruity notes, respectively (Sable and Cottenceau, 1999). Although mold contamination could be implicated as the source of these ketones, surface mold was not observed on any of the experimental cheeses.

Data presented in Table 7 indicate accumulation of 3-hydroxy-2-butanone was significantly reduced in cheese 3. Since 3-hydroxy-2-butanone is produced when cells experience a surplus of pyruvate relative to the need for NAD^+ regeneration, D-HicDH action may inhibit the formation of 3-hydroxy-2-butanone by reducing the available supply of α -keto acids.

The detection of aldehydes in 3-mo Cheddar is significant, since most aldehydes are readily reduced to corresponding alcohols by alcohol dehydrogenases (Engels et al., 1997) and likely facilitated by the strong reducing conditions in hard cheeses. Consistent with this hypothesis, the group of aldehydes accounted for the fewest compounds (Table 7). However, concentrations of individual aldehydes were similar to that of other groups of volatiles such as alcohols, ketones, and fatty acids detected in this study. The strong correlation between production of aldehydes and KG could be the manifestation of active metabolic pathways interconnected via KG or a consequence of shifts in metabolism due to changes in NAD^+/NADH balance within cells (Gottschalk, 1988).

Straight chain aldehydes like nonanal are likely derived via β -oxidation of unsaturated fatty acids (Engels et al., 1997). Documented action of D-HicDH is limited to reduction of straight and branched chain aliphatic and aromatic α -keto acids; hence, the reduced levels of nonanal in cheese 3 and changes in the levels of other aldehydes is not clearly understood. Such effects may, however, be of significance to cheese flavor since nonanal can impart floral-rosy to fruity-citrus flavors, and other aldehydes are generally characterized as grassy aromas (Sable and Cottenceau, 1999).

As stated above, the reducing environment of ripening cheese is believed to facilitate enzymic reduction of methyl ketones, acids, and aldehydes to corresponding

alcohols (Engels et al., 1997). For example, 3-methyl-1-butanol is produced by the reduction of 3-methyl-1-butanal, which is a product of Leu metabolism. The inability of KG to affect 3-methyl-1-butanol levels in cheeses observed in this study is not consistent with the findings of Banks et al. (2001), who reported addition of KG enhanced Leu catabolism and greatly increased 3-methyl-1-butanol levels in cheeses ripened for 6 wk. Our study also found evidence that addition of KG enhanced catabolism of BCAA, as demonstrated by the increased production of 3-hydroxy-2-butanone from Leu or Val. Therefore, the lack of sufficient correlation between KG and accumulation of 3-methyl-1-butanol in this study may be attributed to limitations in other pathways involved in its formation. For example, 3-methyl-1-butanal, a precursor molecule for the production of 3-methyl-1-butanol, can be produced by Strecker reactions.

In contrast, 2-methyl-1-propanol, which is likely derived from catabolism of Val (Engels et al., 1997), increased with addition of KG in both this study and that of Banks et al. (2001). 2, 3-Butanediol also increased with addition of KG and this compound may be obtained from acetoin by dehydrogenation (Engels et al., 1997). 3-Methyl-1-butanol, butanol, and 1-octen-3-ol impart fruity, sweet, and mushroom-like flavors, respectively, in cheese (Adda, 1986). Since 1-octen-3-ol is formed by oxidation of linoleic and linolenic acids (Adda, 1986), it is likely KG plays an indirect role in the increased levels of 1-octen-3-ol that were found in cheeses with added KG. Although the mechanism is not known, decreased levels of 1-octen-3-ol in cheese manufactured with *L.casei* BUS is encouraging because it imparts mushroom-like off-flavors (Milo and Reineccius, 1997).

The most abundant alcohol identified in experimental cheeses was 2-phenethylalcohol, which may be produced by reduction of phenylacetaldehyde or via

Strecker-type reactions and contributes rosy and floral off-flavors to Cheddar (Adda et al., 1982; Dunn and Lindsay, 1985; Gummalla and Broadbent, 2001). Although accumulation of 2-phenylethylalcohol would be expected to rise with increased availability of KG, we detected no influence of KG on the production of this compound, an observation that corresponds with the study of Banks et al. (2001).

Acetic acid was the major fatty acid identified in this study. Short chain fatty acids like acetic acid may be formed from sugar fermentation and citrate metabolism while longer chain fatty acids are likely produced from lipolytic reactions and breakdown of amino acids (Cogan, 1995). The decreased levels of 2-methyl propanoic acid and 3-methyl butanoic acids when KG was added contrasts with the observations of Banks et al. (2001).

Free fatty acids can be released from milk fat due to the action of native milk lipase and microbial enzymes. These free fatty acids may be oxidized to methyl ketones and aldehydes or they may react with alcohols to form esters, which bear sweet and fruity aromas. Esterification can be enzymatically catalyzed or may occur spontaneously between fatty acids and primary alcohols (Engels et al., 1997).

The concentration of acetic acid was significantly influenced by culture system and this effect may be linked to the metabolic energetics of adjunct lactobacilli. Energy is derived via substrate phosphorylation when acetyl phosphate is converted to acetate via the pyruvate-formate lyase pathway (Salminen and von Wright, 1993). The ability of added lactobacilli (*L. casei* 334em) to use this pathway in response to substrate limitation in an anaerobic environment, which are both conditions reflective of cheese ripening, could be responsible for the increased levels of acetic acid in cheese 2. This situation is

very likely since catabolism of amino acids can produce pyruvate, which is the starting point of the above pathway. The capacity of *L.casei* BUS to significantly inhibit accumulation of acetic acid in cheese 3 may be credited to the contribution of enhanced D-HicDH activity towards generation of reducing power in their cells. Consequently, a pyruvate surplus relative to the need for NAD^+ regeneration is diminished and the pyruvate-formate lyase pathway is likely not triggered in their cells. A less significant contribution toward increased accumulation of acetic acid in cheese 2 may come from the metabolism of citrate by adjunct lactobacilli (*L.casei* 334em). In the same way, decreased levels of acetic acid in cheese 3 may be attributed to the ability of *L.casei* BUS to generate reducing power, thereby inhibiting citrate metabolism. The impact of acetic acid on cheese flavor is not clear, but at excessively high concentrations it may cause vinegary off-flavors (Manning and Nursten, 1985).

The detection of both benzaldehyde and phenol are important because they originate from Phe and Tyr, respectively. Phenol can be produced by the action of tyrosine phenol lyase or by Strecker reactions, but evidence for the presence of the former among cheese bacteria is lacking (Fox and Wallace, 1997). On the other hand, Strecker reactions are known to occur in cheese and are important to consider since phenol carries medicinal off-flavor notes (Dunn and Lindsay, 1985). Benzaldehyde is produced by spontaneous degradation of PPA (Groot et al., 1998; Gummalla and Broadbent, 2001). The decrease in concentrations of benzaldehyde observed in cheese manufactured with added KG is in contrast with the study of Banks et al. (2001), who showed accumulation of the former compound in the presence of KG. This variation is likely due to the differences in metabolic activities of bacterial strains used in this study.

For example, in this study adjunct lactobacilli were also used in the manufacture of cheese. Additionally, wild lactobacilli present in the nonstarter bacterial population may also contribute to the observed effects.

The significant reduction of benzaldehyde in cheese 3 suggests enhanced D-HicDH action in *L.casei* BUS in these cheeses likely converted PPA produced from Phe to phenyllactic acid. As mentioned earlier, D-HicDH action may control spontaneous reactions, which convert α -keto acids like PPA to aroma compounds such as benzaldehyde (Groot et al., 1998; Gummalla and Broadbent, 2001). Benzaldehyde imparts a bitter almond and sweet aroma to cheese (Sable and Cottenceau, 1999).

The production of volatile sulfur compounds is vital to development of typical Cheddar flavor (Urbach, 1993). In this study, we identified only dimethylsulfide and dimethylsulfoxide in volatile profiles of experimental cheeses. Dimethylsulfide imparts boiled cabbage and sulfurous aroma to cheese (Sable and Cottenceau, 1999) and research has shown sulfur-containing compounds like dimethylsulfide, dimethyldisulfide, and dimethyltrisulfide are obtained from breakdown of methanethiol, which is a product of Met catabolism (Engels and Visser, 1996). While we observed the accumulation of dimethylsulfide decreased slightly in cheeses with added KG, studies of Yvon et al. (1998) and Banks et al. (2001) reported that addition of KG enhanced Met degradation. It must be noted that the latter works did not report the corresponding increases in production of any Met degradation products. Gao et al. (1998) showed Met breakdown in *Lactococcus lactis* primarily proceeds via transamination even under cheese-like conditions to produce 4-methylthio-2-oxobutyric acid, a precursor of methanethiol. Other reports have suggested few secondary reactions involving cystathionine- β -lyase (CBL)

and cystathionine- γ -lyase (CGL) of lactococci can also make small contributions to the production of methanethiol from Met (Alting et al., 1995; Dias and Weimer, 1998).

Pathways for Met catabolism in lactobacilli are not well studied, but Dias and Weimer (1998) showed ATase, CBL, and CGL may all contribute toward Met degradation by lactobacilli, and perhaps are responsible for the high concentration of dimethylsulfide seen in cheese 2 containing *L.casei* 334em. Levels of dimethylsulfide in cheese 3 were significantly lower than in cheese 2, and this difference may be attributed to metabolic shifts triggered by D-HicDH overexpression cells of *L.casei* BUS present in cheese 3.

The significant differences discovered in sensory responses to sulfur notes relate consistently with data obtained from volatile analysis. For example, cheeses made with KG were perceived as bearing less sulfur notes and contained lower concentrations of dimethylsulfide versus cheeses lacking KG. Since dimethylsulfide was the major sulfur compound identified in these cheeses, this compound may contribute to the sulfur notes perceived by respondents.

Nutty flavor was significantly ($P < 0.1$) lower in cheeses made with KG (Table 5). This effect may be linked to the significantly reduced levels of benzaldehyde detected in the same cheeses. Other volatile nitrogen compounds like pyrazines are attributed with nutty aromas (Moio and Addeo, 1998), but these compounds were not identified in experimental cheeses. Responses to nutty flavor were also significantly altered among cheeses made with different starter combinations. Although previous work has shown *Lactobacillus* adjuncts can enhance nutty flavor notes (Drake et al., 1997), a similar effect was not observed with *L. casei* in this study. Instead, cheeses made with

Lactococcus lactis starter alone were rated with higher nutty flavors than cheeses made with either starter-adjunct combination.

Significant effects of KG and culture treatments were observed in the response to brothy flavor in cheese. Like nutty and sulfur notes, the detection of brothy in cheese was reduced in cheeses made with addition of KG. But, brothy character was enhanced in cheese made with *L. casei* BUS and significantly more in cheese manufactured with *L. casei* 334em.

The intensity of barny notes was significantly reduced in cheese made with KG. Interestingly, only the volatile profile of one cheese containing added KG and manufactured with *Lactococcus lactis* and modified *L. casei* adjunct contained p-cresol, a Tyr catabolite implicated with barny flavor (Dunn and Lindsay, 1985).

Bitter sensations were significantly higher in cheese made with added KG, but this observation is likely related to the inherent bitterness associated with KG. Finally, umami flavor was reported to significantly decrease in cheese with added KG. The occurrence of this note in cheese and other dairy products is attributed to the presence of monosodium glutamate, inosine monophosphate, and guanine monophosphate (Kato et al., 1989).

The contribution of lactobacilli to AAA catabolism and cheese off-flavor development is significant because species such as *L. casei* and *L. helveticus* are widely used as starters or flavor adjuncts, and lactobacilli dominate the NSLAB population in virtually all bacterial-ripened cheeses. Because NSLAB populations exceeded 10^6 CFU per g by 3 mo in all cheeses (Table 6), changes in cheese volatile chemistry and sensory

properties noted in this study cannot be exclusively attributed to the action of *L. casei* 334em and *L. casei* BUS adjunct cultures.

CONCLUSION

Typical Cheddar flavor of reduced fat varieties is tainted by the perception of unclean and off-flavor notes. The metabolism of AAA can be an important source of unclean and off-flavor compounds. It is recognized that AAA catabolism by lactococci and lactobacilli in the cheese environment is almost exclusively initiated by transaminase. The α -keto acids produced from many of these reactions are chemically labile and may degrade spontaneously into a variety of potent aroma compounds (Gao et al., 1997; Groot et al., 1998; Gummalla and Broadbent, 1999; Gummalla and Broadbent, 2001). Previous research by our group on AAA catabolism by *L. casei* under cheese-like conditions revealed these bacteria could produce dehydrogenases such as D-HicDH that convert α -keto acids into more stable α -hydroxy acid derivatives (Gummalla and Broadbent, 1999; Gummalla and Broadbent, 2001). Because spontaneous degradations of α -keto acids derived from AAA catabolism leads to the production cheese off-flavor compounds (Gao et al., 1997; Gummalla and Broadbent, 2001), it was our hypothesis that D-HicDH overexpression may limit off-flavor development. To pursue this hypothesis we investigated the effect of D-HicDH overexpression by a *L. casei* adjunct culture on the volatile and sensory profiles of 50% reduced-fat Cheddar cheese made with and without added KG.

Results showed D-HicDH overexpression or addition of KG influenced bacterial metabolism and the accumulation of volatile compounds in cheese. With respect to the

addition of KG, our results are similar to those reported by Yvon et al. (1998) and Banks et al. (2001) with few exceptions. The differences observed between studies may be attributed to differences among strains used. The influence of KG on the accumulation of cheese volatiles is likely linked to its direct participation in transamination reactions.

Overexpression of D-HicDH in *L.casei* BUS influenced metabolism of ketones, aldehydes, alcohols, acids, dimethylsulfide, and benzaldehyde. One hypothesis for the ability of D-HicDHs to influence the levels of such a diverse group of compounds may be linked to the potential of D-HicDH reactions to generate reducing power in bacterial cells by reoxidizing NADH produced from other metabolisms. While this feature may be advantageous to LAB in the ability to maintain a redox balance within their cells, this situation can also trigger bacteria to alter metabolism of other substrates resulting in a variety of end products. Furthermore, many cellular chemicals act as metabolic intermediates that interconnect a variety of pathways, thereby expanding the potential of D-HicDH to influence accumulation of a large number of compounds. Cheeses manufactured with *L. casei* BUS contained reduced levels of products of chemical decomposition, but these cheeses also contained diminished levels of beneficial flavor notes. For example, accumulation of benzaldehyde was significantly lower in cheese made with the D-HicDH-overexpressing strain of *L. casei*. However, levels of other compounds like dimethylsulfide and 3-hydroxy 2-butanone, which are believed to contribute to typical Cheddar notes, also diminished in these cheeses.

This disadvantage may be addressed by the examination of other members of this class of enzymes that display more narrow substrate specificity. Hydroxy acid

dehydrogenase activity has been noted in *L. curvatus* (Hummel et al., 1988), *L. bulgaricus* (Bernard et al., 1994), *L. confusus* (Lerch et al., 1989), *Enterococcus faecalis* (Yamazaki and Maeda, 1986), *Pediococcus acidilactici* (Delcour et al., 1993), and *Leuconostoc* spp. (Hummel et al., 1985). In addition, several genes encoding homologs to L-HicDH and L-lactate dehydrogenases were recently located in the *Lactococcus lactis* genome (Bolotin et al., 2001). These observations suggest that hydroxy acid dehydrogenases are widespread among LAB, and enzyme screening programs may identify enzymes that possess a more limited specificity for aromatic α -keto acids.

Alternatively, substrate specificity can be altered, by modifying the region of the enzyme that is involved in substrate recognition. Substrate binding amino acids and other residues involved with the NAD⁺ binding domain are conserved in this group of enzymes and their action is closely related to reaction mechanisms of lactate dehydrogenases. Feil et al., (1994) introduced deletions into the L-hydroxyisocaproate dehydrogenase gene by site-directed mutagenesis and showed deletion variants possessed altered substrate specificity.

The overall effect of D-HicDH overexpression in this study was observed to be a diminution of both beneficial and detrimental flavor notes. But the above strategies can make available useful D-HicDH enzymes with selective substrate specificities.

Finally, because experimental cheeses contained a growing population of wild lactobacilli, their contribution to the observed effects cannot be ignored. Therefore, the ability to monitor NSLAB diversity in experimental cheeses may provide valuable information and a clearer interpretation of the results obtained in this study.

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CHAPTER V

CONCLUSIONS AND SUMMARY

In the last few decades, consumption of dairy products has risen dramatically. Today, this growing demand is fulfilled by an extensive assortment of milk products, including reduced-fat varieties. Manufacture of high-quality, low fat cheese for the growing number of consumers who wish to decrease their level of dietary-fat intake has become a national priority. Unfortunately, most reduced-fat Cheddar cheeses suffer from poor flavor intensity, a propensity for off-flavor development, and inferior body and textural qualities. These concerns have stimulated a significant amount of interest in the biochemistry of cheese ripening and flavor development. Although a considerable amount of new knowledge is available, a comprehensive understanding of cheese ripening and flavor development is lacking.

Amino acids, which were once believed to be directly involved with flavor development, are currently the subject of study as precursors to important flavor compounds (Urbach, 1995). Some aromatic compounds like indole, skatole, *p*-cresol, phenol, and phenethanol that have historically been associated with unclean sensations in cheese are derived from catabolism of AAA of cheese bacteria (Dunn and Lindsay, 1985). Microbial catabolism of AAA is well known in other bacteria but limited in cheese microorganisms. Research in LAB has shown that the starter organism *Lactococcus lactis* initiates degradation of AAA by an aromatic aminotransferase under cheese-ripening conditions (Gao et al., 1997). Previous study by our laboratory revealed lactobacilli are also able to catabolize Trp via transamination reactions (Gummalla and

Broadbent, 1999). Our group is interested in the contribution of lactobacilli to AAA catabolism and cheese off-flavor development because species such as *L. casei* and *L. helveticus* are widely used as starters or flavor adjuncts, and members of this genus also dominate populations of NSLAB (adventitious) in virtually all ripened cheese varieties (Lee et al., 1990; Peterson and Marshall, 1990).

This study investigated Tyr and Phe catabolism by *L. casei* and *L. helveticus* cheese flavor adjuncts under cheese-ripening conditions. Results showed that both microorganisms utilize a similar pathway for Tyr and Phe catabolism under cheese-ripening conditions, wherein AAA are catabolized by reversible, successive, and constitutively expressed transamination and dehydrogenation reactions. Additionally, α -keto acids produced by transamination of AAA were unstable and subject to spontaneous degradations into off-flavors. However, these bacteria also produce dehydrogenases such as D-HicDH that convert α -keto acids into more stable α -hydroxy acid derivatives (Gummalla and Broadbent, 1999; Gummalla and Broadbent, 2001).

Because spontaneous degradations of α -keto acids derived from AAA catabolism leads to the production of cheese off-flavor compounds, it was our hypothesis that D-HicDH overexpression may help to control off-flavor development.

Results obtained in this study suggest D-HicDH overexpression and addition of α -ketoglutarate altered cheese bacterial metabolism and influenced the accumulation of volatile compounds. However, wild NSLAB were also present in all experimental cheeses and they may have contributed to these observations. With respect to the addition of α -ketoglutarate, our results are similar to those reported by Yvon et al. (1998) and Banks et al. (2001) with few exceptions.

Overexpression of D-HicDH in *L. casei* BUS influenced metabolism of ketones, aldehydes, alcohols, acids, and the aromatic compound, benzaldehyde. One hypothesis for the ability of D-HicDH to sway the levels of such a diverse group of compounds may be linked to the potential of D-HicDH reactions to generate reducing power in bacterial cells. Alternatively, many cellular chemicals act as metabolic intermediates that interconnect a variety of pathways, thereby expanding the potential of D-HicDH to influence accumulation of such a large number of compounds.

Overexpression of

D-HicDH appeared to divert α -keto acids into more stable compounds but the overall effect is observed to be a diminution of both beneficial and detrimental flavor notes. The role of D-HicDH in preventing spontaneous reactions is evident in the observation that accumulation of benzaldehyde was significantly lower in cheese made with over-expressing strain of *L. casei*. However, levels of other compounds like dimethylsulfide and 3-hydroxy-2-butanone, which are believed to contribute to typical Cheddar flavor, also diminished in these cheeses.

The above poses an important limitation in using this D-HicDH overexpression system to control spontaneous production of off-flavor compounds. This situation may be addressed by identification of related enzymes with narrower substrate specificity, and the plausability of this approach is supported by enzyme studies and genome sequence data that show hydroxy acid dehydrogenases are widely distributed among LAB. If suitable enzymes cannot be found, it may be possible to alter the substrate specificity of existing enzymes by site-directed mutagenesis of substrate recognition sites. Evidence to support this approach comes from the work of Feil et al. (1994), who showed deletion

mutations in the L-hydroxyisocaproate dehydrogenase gene produced enzyme variants with altered substrate specificity.

Finally, because experimental cheeses contained a growing population of wild lactobacilli, their contribution to the observed effects cannot be ignored. Therefore, experiments to monitor NSLAB diversity in experimental cheeses may facilitate data interpretation in future studies of this type.

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APPENDIX

February 23, 2002

Sanjay Gummalla
900 North Stuart Street, #1111
Arlington, VA-22203
(703) 465-8327/sanjayg@cc.usu.edu

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Savoy, IL 61874

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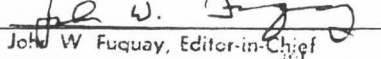
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2 / 26 / 02

07-02-02

Dr. Joanne E. Hughes
Research Assistant Professor, Biology
Utah State University
Logan, UT-84322
435-797-3552

Dear Dr. Hughes,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences department at Utah State University. I hope to complete in the summer of 2002.

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I hope you will be able to reply immediately.

Thank you for your cooperation,

Sanjay Gummalla

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Overexpression of Lactobacillus casei D-Hydroxyisocaproic acid dehydrogenase: Effects on cheese volatiles and sensory properties. Co-authored by S. Gummalla, J. E. Hughes, M. E. Johnson, S. Rankin, M. A. Drake, and J. R. Broadbent.

Signed

Joanne E. Hughes

07-02-02

Dr. Mark Johnson
Wisconsin Center for Dairy Research
1605 Linden Dr.
Madison, WI 53706-1565
608-262-0275

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Signed



07-02-02

Dr. Scott A. Rankin
Assistant Professor, Food Science
A203, Babcock Hall,
University of Wisconsin-Madison
Madison, WI 53706-1565
(608) 263-2008

Dear Dr. Rankin,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences department at Utah State University. I hope to complete in the summer of 2002.

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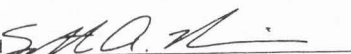
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Signed



07-02-02

Dr. Mary Ann Drake
Assitant Professor, Food Science
North Carolina State University
Raleigh, NC 27695-7624
(919) 513-4598

Dear Dr. Drake,

I am in the process of preparing my dissertation in the Nutrition and Food Sciences department at Utah State University. I hope to complete in the summer of 2002.

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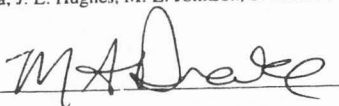
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Signed



CURRICULUM VITAE

Sanjay Gummalla
(April 2002)

CAREER OBJECTIVE:

To be part of a challenging and innovative technology group in the life sciences.

EDUCATION:

BS in Agriculture, Acharya N. G. Ranga University of Agricultural Sciences, Hyderabad, Andhra Pradesh, India. (09/1993) GPA: 8.52 (10.0=A). MS in Nutrition and Food Sciences, Utah State University, Logan, Utah. (12/1998), Thesis entitled: Tryptophan catabolism by *Lactobacillus* spp.: Biochemistry and implications on flavor development on reduced-fat Cheddar cheese. PhD in Nutrition and Food Sciences, Utah State University, Logan, Utah. (expected 04/2002) Grad GPA: 3.70 (4.0=A). Dissertation entitled: Aromatic amino acid catabolism by *Lactobacillus* spp.: Biochemistry and contribution to cheese flavor development.

EXPERIENCE:

Graduate Research Assistant, Department of Nutrition of Food Science, Utah State University (1993-2001).

Post Doctoral Associate, Joint Institute of Food Safety and Applied Nutrition, University of Maryland-College Park, and the Food and Drug Administration (December 2001 to present).

PUBLICATIONS:

Gummalla, S. and J. R. Broadbent, 1999. Tryptophan catabolism in *Lactobacillus casei* and *Lactobacillus helveticus* cheese flavor adjuncts. J. Dairy Science, 82: 2070-2077.

Gummalla, S. and J. R. Broadbent, 1999. Tyrosine and Phenylalanine catabolism by *Lactobacillus* cheese flavor adjuncts. J. Dairy Science, 84: 1011-1019.

AWARDS:

1999, Niranjana, R. Gandhi and Josephine N. Gandhi Fellowship, Department of Nutrition and Food Sciences, Utah State University.

1997 and 1996, E.L Inez and Waldron Endowment Scholarship, Department of Biotechnology, Utah State University.

1996, Second prize; Graduate student paper competition, Annual meeting of the American Dairy Science Association.

1996 and 1995, Second prize; Graduate student paper competition, Annual meeting of the Intermountain branch of the American Society for Microbiology.